

**CELLULAR EFFECTS OF ENAMEL MATRIX
DERIVATIVE ARE ASSOCIATED WITH
SPECIFIC PROTEIN COMPONENTS**

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CELLULAR EFFECTS OF ENAMEL MATRIX DERIVATIVE ARE ASSOCIATED WITH SPECIFIC PROTEIN COMPONENTS

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Background: Emdogain[®], or enamel matrix derivative (EMD), is a preparation of matrix proteins derived from developing porcine teeth. Although EMD has been shown to enhance both soft tissue healing and regeneration of the periodontium, the mechanism of this action is still unknown. It is assumed, but not yet proven, that amelogenin, the most abundant protein in EMD, is the protein primarily responsible for the effects of EMD. The purpose of this study was to fractionate EMD and associate specific cellular effects with specific protein components.

DEDICATION

I would like to dedicate this to my dear wife and best friend, ~~FAITH~~. Her support, love, encouragement, and understanding have truly been the “wind beneath my wings.” I am so grateful for her dedication, patience, and commitment, as well as the many sacrifices she has made during the last three very busy and demanding years. Words cannot even begin to describe the love and appreciation I feel for her. Much of the time she has had to be both mom and dad to our six sons; To each of my wonderful boys, ~~DAVID, JACOB, JESSE, JONAS, JOSHUA, and JUDAH~~ for sacrificing special time with dad and trying to understand the many hours of study and writing required to complete this project and many others associated with the residency; To my parents and siblings, for their encouraging calls, visits, support and prayers. Finally, to my Lord and Savior, Jesus Christ, who has sustained, comforted, and given me hope in my most difficult and trying hours. Without Him none of this would even have been possible.

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**THE VIEWS EXPRESSED IN THIS ARTICLE ARE
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Methods and Materials: Thirty milligrams of EMD were fractionated over a Sephadex G-100 column. Forty-five 7ml fractions were collected, desalted, lyophilized, and resuspended in 10mM acetic acid. The amount of protein was determined and each fraction adjusted to 25µg protein/ml with cell culture media. Cell proliferation was examined in osteoprogenitor (C2C12) and human microvascular endothelial cells (HMVEC). Noggin-sensitive alkaline phosphatase activity (C2C12) and angiogenesis (HMVEC) were evaluated as markers for differentiation. Polyacrylamide gels containing protein substrate (denatured type I collagen) were used to determine collagenolytic activity in each fraction.

Results: EMD fractionated into three major protein peaks associated with the column void volume, the 50 kDa and the 10 kDa molecular weight proteins. The void volume peak represents proteins greater than 100 kDa molecular weight while the 50 kDa and 10 kDa peaks most likely represent amelogenin and fragments, as well as other minor proteins in the mixture. Proliferation activity was associated with the 50 kDa and 10 kDa proteins peaks for both osteoprogenitor and microvascular cells. Differentiation of osteoprogenitor cells, as indicated by alkaline phosphatase activity, was stimulated by protein fractions between the void volume and 50 kDa peaks. This activity was inhibited by prior treatment of the fractions with noggin, indicative of bone morphogenetic protein (BMP) activity rather than amelogenin activity. Angiogenic activity (HMVEC) was associated with the same fractions that stimulated proliferation. Collagenolytic activity was associated with the void volume, as well as with proteins in the 68 kDa and 25-40 kDa molecular weight regions.

Conclusions: EMD stimulated multiple activities in more than one cell type, important because of the multiple cell types involved in the regenerative process. These cellular

activities are apparently not associated with a single protein species, i.e. amelogenin, because the activities are associated with proteins of differing molecular weights. Differentiation of osteoprogenitor cells is probably the result of BMP(s) present in the EMD mixture and not the result of amelogenin activity because it fractionated in a different molecular weight region from amelogenin, and was inhibited by noggin, a known antagonist of BMP-2, -4, and -7. This suggests that cooperation of multiple growth factors may be important for successful periodontal regeneration. EMD also stimulates proliferation and differentiation of microvascular endothelial cells, activities not previously reported, and these activities are associated with the fractions containing amelogenin. Finally, EMD was shown to contain collagenolytic activity, also not previously reported, that is capable of degrading type I collagen. This proteolytic activity was manifest in both the 68 kDa and 25-40 kDa molecular weight regions, consistent with MMP-2 and/or MMP-20, both known to digest amelogenin. This is important for both matrix degradation and cell migration during regeneration of the periodontal tissues. Although the multiple cellular effects of EMD cannot be ascribed to any single protein species, this study suggests that combined activities may be necessary for successful periodontal regeneration.

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I. INTRODUCTION AND LITERATURE REVIEW

A. INTRODUCTION

Periodontitis, one of the most common reasons for tooth loss in the United States today, occurs due to the loss of the supporting structures of the periodontium, specifically, cementum, periodontal ligament (PDL) and alveolar bone. This disease has a prevalence of between 35-70% of the U.S. population, and even though the disease may not be curable, it is treatable. For years, the goal of therapy was to eliminate the etiologic factors in order to stop the continued loss of the supporting structures. In the last decade, the advances in periodontal therapy have led to the rationale of not only trying to stop progression of the disease and maintain what is left of the attachment apparatus, but also to trying to regenerate what has been lost.

Regeneration has been approached and attempted in many ways with varying degrees of success and predictability. This is because successful periodontal regeneration requires the integration of multiple tissue interactions in order to build or rebuild the entire periodontal attachment apparatus. Materials such as autogenous and allograft bone have been used to create a scaffold on which resident cells can grow and provide protein factors to help stimulate host cell growth into the defect. There are, however, varying theories as to what target cells should be stimulated in the host. Melcher initially suggested that cells from the periodontal ligament hold the potential for regenerating the attachment apparatus (Melcher, 1976), but a few years later modified his theory to include bone cells along with cells from the periodontal ligament. Meyer states that pluripotential periodontal ligament cells differentiate into cells of cementogenic, osteogenic and fibroblastic lineage (Meyer,

1986). More recent studies on the other hand suggest that the formation of acellular cementum is the key tissue in the development of a functional periodontium (Hammarström *et al.*, 1997) and that periodontal ligament and alveolar bone development is associated with the formation of acellular cementum. In either case, these theories led to the use of barrier membranes in an attempt to exclude epithelial and gingival fibroblasts from entering wound sites, thus giving these precursor cells the opportunity to “regenerate” the attachment apparatus. This treatment modality of regeneration is commonly known as guided tissue regeneration (GTR).

GTR has been proven successful both clinically and histologically and is an important part of periodontal therapy. The concept of GTR has also progressed over the years from using materials such as non-resorbable membranes, which require a second-stage surgical procedure for removal, to using resorbable porcine and bovine collagen membranes, which degrade over time. Even though these modalities of treatment were successful, complete regeneration was not always predictable or consistent. To try and increase the predictability of regeneration, GTR was used in combination with osseous autografts and allografts, as well as with xenografts and alloplasts. Even though these combination approaches improved regeneration in certain situations, the goal of predictable and complete regeneration has yet to be achieved.

Current research on regeneration has focused on attempting to regenerate the attachment apparatus by mimicking the events that occur during the initial embryogenesis of the periodontal tissues (Gestrelius, 2000). Root formation is primarily the result of the down growth of the apical extension of the enamel organ, known as Hertwig’s epithelial root sheath (HERS). It is thought that Hertwig’s epithelial root sheath is responsible for

depositing enamel matrix proteins on the root surface (Slavkin and Boyde, 1975; Slavkin, 1976) that may be the stimulus initiating cementum formation. *In vitro* studies show that acellular cementum is formed when either endogenous or exogenous enamel matrix is exposed to dental follicle cells (Hammarström, 1997b).

Because of the complexity of the interactions of the many cell types, growth factors and proteins associated with nascent tooth formation, there has been much research into how these elements function individually and in concert to potentially regenerate a diseased periodontium. With the discovery that HERS secretes enamel matrix proteins in association with root and acellular cementum formation, more of today's focus has been on using enamel matrix proteins to induce host cells to regenerate new cementum, periodontal ligament and alveolar bone. These enamel matrix proteins are available today in a material known as enamel matrix derivative (EMD).

Enamel matrix derivative, commercially known as Emdogain® was originally produced and distributed by Biora™ AB, Malmö, Sweden. The company has been purchased and now operates as Straumann Biologics Division, Waltham, MA. Emdogain® is now marketed by Straumann as a surgical adjunct for use in regeneration. According to the company, indications for use include such areas as intrabony defects and gingival recession, as well as shallow class II furcation defects. All situations involve attempts to regenerate periodontal tissues that have been lost due to disease. The effectiveness of EMD has been proven both clinically and histologically in both animal and human models. Clinically, EMD has been used in many situations with promising results. When EMD is used in conjunction with open flap debridement compared to open flap debridement alone the outcome is better; When GTR is compared to EMD the results have also been comparable. The combined use

of EMD with particulate grafting materials, whether allograft or xenograft, has also received increasing amounts of attention in the quest for complete and predictable regeneration. The results have not been decisive, but an obvious trend appears to be evident favoring better results when the two materials are combined.

Even though there is mounting evidence for the successful regenerative potential of EMD, the mechanism of action by which EMD exerts its effects on the tissues involved in the regenerative process continues to elude investigators. It is truly a case in which the preliminary research necessary to understand the mechanism of action leading to the desired results was not accomplished prior to FDA approval for its clinical use in the field of periodontics. The observed clinical success of the product has prompted investigators to search for the reason(s) behind the clinical success. Much of that research has focused on amelogenin, the major protein component of EMD. The underlying question still to be answered is: are the observed beneficial effects of EMD a result of amelogenin or some other component(s) in the mixture?

The ideal way to answer this question would be to separate the proteins (i.e. amelogenin) contained in the commercially available mixture of EMD, apply them to multiple cell types, and then assay for activities, such as proliferation and differentiation, known to be associated with EMD. Performing such experiments and then associating the activities with the various protein components of the mixture would help determine and clarify whether or not the observed activities and clinical success are the result of a single protein component (i.e. amelogenin).

The goal of this research was to separate the various proteins contained in EMD and associate the known cellular activities with the proteins contained in EMD in order to determine if amelogenin is the protein responsible for its regenerative properties.

B. LITERATURE REVIEW

1. PERIODONTAL DISEASE

Periodontics is the specialty of dentistry involved in dealing with the tissues near, around, or investing (peri) the teeth (odontics). Those tissues, collectively known by the term "periodontium", primarily involve the gingiva, alveolar mucosa, cementum, periodontal ligament, and alveolar and basal bone (AAP, 2001). When these tissues become diseased in any way, the condition is known by the broad term of periodontal disease. This disease and many of its forms have been classified and reclassified over the years in an attempt to categorize and describe the nature, extent, and severity of the disease process that is occurring (Annals of Periodontology, 1999).

Periodontal disease currently is divided into two broad categories based upon the amount of tissue destruction that occurs and the reversibility of the diseased state. Those terms are gingivitis and periodontitis. Gingivitis is the less severe of the two disease states and as the term implies, is an inflammatory condition affecting primarily the gingiva and occurs without any corresponding loss of attachment. It occurs over time as a result of the primary etiologic factor, bacterial plaque (Löe *et al.*, 1965; Socransky and Haffajee, 1991), and progresses through various histologic stages (Page and Schroeder, 1976). This disease process is considered reversible, and once the bacterial plaque is removed, a normal state of gingival health can be achieved as long as no attachment loss has occurred. Once the

inflammatory process progresses to the point of losing underlying supporting tissue, i.e. bone, periodontal ligament, and cementum, the irreversible diseased state known as periodontitis exists. However, the progression of gingivitis to periodontitis and the accompanying loss of attachment do not occur universally in every person (Löe *et al.*, 1986; Page and Schroeder, 1981). In fact, according to Brown *et al.* (1988), the prevalence of gingivitis in the United States was approximately 50% while that of periodontitis was 36%.

The variability in an individual's progression from gingivitis to periodontitis requires more than the mere presence of bacterial plaque. Periodontitis is truly multifactorial in nature. Host susceptibility (Greenstein and Lamster, 2000; Kornman *et al.*, 1997; Seymour, 1991), including an individual's immunologic state, the presence of "favorable or non-pathologic bacteria", calculus, smoking, occlusion, anatomic anomalies, age and genetics, have all been shown, to serve as possible modifying or contributing factors. Each affects the detrimental shift from gingivitis to periodontitis and a corresponding loss of attachment to one degree or another.

The dentogingival attachment apparatus around healthy teeth and its corresponding dimensions was originally described by Gargiulo *et al.* (1961) in which they found a mean sulcus depth of 0.69mm, and mean junctional epithelium and connective tissue attachments of 0.97mm and 1.07mm respectively. In periodontitis, the underlying connective tissue, bone, and PDL are destroyed, resulting in an apical migration of the junctional epithelium, with accompanying clinical attachment loss (CAL) and increased probing depths (PD). Deep probing depths also provide a conducive environment that potentiates a bacterial shift from the predominantly gram-positive flora associated with health to the predominantly gram-negative flora necessary to cause disease. If left untreated, the likelihood of continued CAL

and tooth loss is extremely high (Løe *et al.*, 1986; Becker *et al.*, 1979). The destructive nature of the disease may produce osseous defects around teeth, some of which may be amenable to regenerative therapy.

2. PERIODONTAL TREATMENT

For many years, the goal of periodontics was to halt CAL leading to tooth loss by the removal of the primary etiologic factor, bacterial plaque. In doing this, as well as in attempting to eliminate or at least decrease the number of secondary etiologic factors, it was hoped that an individual could surpass a certain threshold in which an equilibrium between continued destruction and a reduced but healthy periodontium would exist. At best, healing by repair with a long junctional epithelium was all that could be hoped for. This objective was realized by using both non-surgical and surgical means of therapy to eliminate both the etiologic factors and decrease the pocket depths.

A non-surgical approach with scaling and root planning is routinely provided first and is a proven method of eliminating bacterial plaque, and decreasing probing depths, inflammation and tooth loss (Hujoe *et al.*, 2000; Morrison *et al.*, 1980; Lindhe *et al.*, 1982; Hung and Douglass, 2002), however, the inability to completely remove accretions or reduce the probing depths to levels that can be adequately maintained by both the patient and dental professionals, many times requires a surgical approach. For many years the only surgical approach available was one that was resective in nature. In the early years it was primarily accomplished through soft tissue resection or gingivectomy (Orban, 1942; Goldman 1951; Glickman, 1956; Waite, 1975), but this only partially solved the problem of deep probing depths. Many times the deeper probing depths were not only due to soft tissue but to

underlying bony defects, therefore osseous resection was applied (Schluger, 1949). This was an effective means in reducing pockets, and eliminating etiologic factors as well as facilitating maintenance by both the patient and dental professional but not without the drawbacks of increased attachment loss from recession and increased root sensitivity. This treatment modality offering the compromise of a "reduced but stable periodontium" was acceptable for many years in periodontics and is an acceptable means of treatment even today in the 21st century. However, with the discovery that the periodontium can indeed be regenerated under certain circumstances, the once held belief that periodontitis was an irreversible process is fast becoming a part of historical periodontics.

3. PERIODONTAL REGENERATION

In 1970, a landmark article by Schallhorn *et al.* (1970) demonstrated clinical supracrestal bone growth in "no wall", one-wall, and two-wall defects using fresh frozen bone from the iliac crest. From this point on, the quest of treating periodontitis by means of regeneration, an additive technique, instead of a resective technique, became the ultimate goal. The fact that the magnitude of supracrestal bone growth in this landmark study has never been reproduced is indicative of one of the greatest challenges inherent in regeneration, that of predictability. Even though there is histologic proof of principle (Dragoo and Sullivan, 1973; Bowers *et al.*, 1985, 1989), that both autogenous bone and allograft may achieve true regeneration of bone, PDL and cementum, the fact remains that there are inconsistencies and dissenting opinions (Becker *et al.*, 1995).

Attempts at increasing the predictability of regeneration have led to newer treatment modalities such as that initially described by Gottlow *et al.* (1986) as guided tissue

regeneration (GTR), in which barrier membranes are used in an attempt to exclude the epithelium in order to give precursor cells from bone and PDL time to initiate the regenerative process. GTR has proven to be successful using both non-resorbable and resorbable barrier membranes (Cortellini *et al.*, 2004; Gottlow *et al.* 1986; Nyman *et al.*, 1982). In some instances (furcation defects), the combined use of both GTR and allograft has proven to be more conducive to regeneration while at other times (intra-bony defects), the combination therapy has made no difference (Murphy and Gunsolley, 2003). The fact remains that a method of achieving predictable and complete regeneration of bone, PDL and cementum, 100% of the time has yet to be found. Much of today's search for such a material or adjunct has turned to the use of biologics such as growth factors and enamel matrix proteins.

4. ENAMEL MATRIX DERIVATIVE

A current and increasingly popular adjunct used in today's treatment of periodontal disease is Enamel Matrix Derivative (EMD), commercially known as Emdogain®. This section will discuss its development, indications for use, composition, and some of the controversies surrounding the nature of attachment, possible mechanisms of action, and current research to date.

EMD is a mixture of freeze-dried enamel matrix proteins derived from the tooth germs of fetal porcine teeth. It was approved for clinical use by the FDA in 1997 and was originally produced and distributed by Biora™, a Swedish company. In 2004, however, it was purchased by and began to be marketed commercially by Straumann™, a company widely known for their implant system. As part of its introduction for use in periodontics, a

whole issue of *The Journal of Clinical Periodontology*, (Vol 24, Number 9, 1997) was devoted to research articles demonstrating its effectiveness both *in vitro* and *in vivo*. The producers, past and present, claim the following may be indications for its clinical use: intrabony defects, gingival recession, and shallow class two furcation defects. Furthermore, it has also been used clinically in treating avulsed teeth, endodontic surgery (failed RCT), and in bony defects from third molar extraction sites.

EMD has demonstrated the ability to enhance regeneration when applied in conjunction with periodontal surgery showing the formation of new acellular cementum, bone and periodontal ligament (PDL) in one human subject (Heijl, 1997) and in monkeys with dehiscences (Hammarström *et al.*, 1997). Another study showed that the use of EMD in periodontal defects has a superior defect fill compared to surgical debridement alone (Froum *et al.*, 2001). It also selectively enhances the proliferation of PDL cells but not of epithelial cells (Gestrelius, 1997). This last effect is due primarily to its cytostatic effect against epithelial cells in suppressing the down growth of junctional epithelium onto the root surfaces (Kawase *et al.*, 2000). Contrasting research suggests that when the enamel proteins are secreted by the HERS, they are only secreted in the apical portion of the root and are not present in the region of acellular cementum growth during root development (Fong and Hammerström, 2000). Other studies show that the regeneration of acellular cementum is not a consistent finding (Sculean *et al.*, 1999; Yukna *et al.*, 2000). In spite of these studies reporting clear histological differences, the number of successful clinical studies evaluating different applications and/or combinations of uses continues to grow.

The promising clinical results of using EMD have only grown since it was first shown in 1997 that the clinical parameters after surgery were better using EMD than for open flap

debridement alone (Heijl *et al.*, 1997; Okuda *et al.*, 2000; Francetti *et al.*, 2004). When comparing EMD to GTR using resorbable membranes, no superiority or significant differences of one treatment over the other could be demonstrated (Sculean *et al.*, 2004, Silvestri *et al.*, 2000), but the use of EMD did appear to decrease the percentage of post-operative complications (Sanz *et al.*, 2004). This finding gives credence to the idea that there is an accelerated soft tissue healing response associated with its use. One study attempting to explain the mechanism by which the better soft tissue healing that occurred when using EMD stated that it was due to its ability to resolve the post-operative inflammation more quickly (Wennström and Lindhe, 2002). Once again, however, a contrarian study implied that there is no difference in soft-tissue wound healing following periodontal surgery with the use of EMD (Hagenaars *et al.*, 2004). One meta-analysis evaluating EMD's use in regenerating intrabony defects found that even though there was a wide range of variability in the outcome, the average clinical attachment level gain of 3.2mm and corresponding average probing depth reduction of 4mm (Kalpidis and Ruben, 2002) was still quite significant. Another important meta-analysis was conducted by the 2003 Workshop on Contemporary Science in Clinical Periodontics in which the evidence for using EMD for repair and regeneration was evaluated. The authors concluded that the evidence does support its use for improving CAL's and reducing PD's (Giannobile *et al.*, 2003). The fact that EMD is non-space maintaining has also led some researchers to try combining various particulate grafting materials with EMD. One *in vivo* study, which combined DFDBA with EMD, found that it had an increased osteogenic effect in the mouse calf muscle (Boyan *et al.*, 2000). A recent clinical trial combining these same two materials found that the greatest advantage of using EMD + DFDBA versus DFDBA alone was a greater percentage of bone fill, 74.9% and

55.3%, respectively (Gurinsky *et al.*, 2004). When combining xenografts, specifically Bio-Oss, with EMD, the results have been mixed with some authors finding an additional benefit (Zucchelli *et al.*, 2003; Valasques-Plata *et al.*, 2002; Lekovic *et al.*, 2001), while other authors have not (Scheyer *et al.*, 2002; Sculean *et al.*, 2002). Even though EMD continues to be used clinically by many periodontists with encouraging results, controversy still exists and there are many questions left unanswered.

Many *in vitro* studies attempt to answer the questions that continue to arise such as what are the cellular effects of EMD and are they cell dependent. One such study shows that EMD has the effect of both stimulating proliferation and/or differentiation depending on the cell type (Schwartz *et al.*, 2000). Another suggests that EMD inhibits the growth of gram-negative periodontal pathogens (Spahr *et al.*, 2002), while another states, that the antibacterial properties on the specific periodontal pathogen, *Porphyromonas gingivalis*, is more due to the carrier, propylene glycol alginate, than the EMD itself (Newman *et al.*, 2003).

Other effects of EMD appear to include stimulating the attachment and spread of periodontal ligament cells as well as the release of transforming growth factor β (TGF- β) (Van der Pauw *et al.*, 2000). Several studies focus on the major protein in EMD, amelogenin, as a cellular adhesion protein potentially responsible for its therapeutic effects (Huang *et al.*, 2002) and as the molecule that contains integrin binding sites for cellular attachment (Lyngstadaas *et al.*, 2001; Suzuki *et al.*, 2001). Some investigators have shown that it is not the amelogenin in the EMD that is causing the effects; rather, it is some type of bone morphogenetic protein (BMP) (Iwata *et al.*, 2002) or transforming growth factor (Kawase *et al.*, 2001).

Even with the known and well-documented activities of EMD, there still seems to be conflicting reports in the literature. Multiple markers such as alkaline phosphatase activity and osteocalcin are used to identify cells with osteoblast phenotypes as well-differentiated osteoblasts (Aubin *et al.*, 1995 and 1998). Several studies have found that these markers are present and show that EMD does promote cellular differentiation (Gestrelus 1997; Schwartz *et al.*, 2000; Ohyama *et al.*, 2002), whereas another study states that osteocalcin is not present (Hakki *et al.*, 2001) and does not promote differentiation in either osteogenic or myofibroblastic cell lines (Chano *et al.*, 2003). There are many explanations for these differences but one is the difference in cell lines used. Of particular interest to this research project are the studies by Ohyama (2002) which uses the pluripotential C2C12 mesenchymal cell lines from mouse calf muscle and arrives at the same conclusion: that EMD inhibits the differentiation of the C2C12 cells into myoblasts and promotes differentiation into osteoblast and chondroblast cells. Also of particular interest to this research is a study in which a ST2 mouse bone marrow stromal cell line was used in conjunction with porcine enamel extracts (not Emdogain®) and then fractionated in order to isolate the osteoinductive portion. The osteoinductive fraction containing the principal enamel protein amelogenin, however, did not stimulate any alkaline phosphatase activity in the ST2 cells. Using the recently discovered protein noggin, found to antagonize the functions of BMP's, the authors showed that the osteogenic activity of the enamel extracts was attributed to BMP instead of amelogenin (Iwata *et al.*, 2002).

The fact that EMD stimulates cellular proliferation and differentiation seems to be well established and accepted by most researchers today. However, the nature of the

regeneration that occurs and its mechanism of action whether, associated with amelogenin, BMP or some other protein, are still largely unknown and very controversial.

5. AMELOGENIN

Amelogenin is the major protein component of EMD making up 90% of the enamel matrix produced by the epithelial-derived ameloblasts (Gestrelus *et al.*, 2000). Other proteins such as amelin (also called ameloblastin or sheathlin), enamelin, and tuftelin, along with proteases and albumin complete the known enamel matrix. Amelogenin is a 20-25 kDa hydrophobic molecule in the enamel matrix whose main functions during enamel formation include mineral ion binding as crystal precursors, control and support of growing crystals, and protection of the mineral phase (Robinson *et al.*, 1998). This protein is nearly completely homologous among all higher vertebrates with only 4% of the gene transcript bases differing between humans and pigs (Gestrelus *et al.*, 2000). The high similarity between porcine and human amelogenin nearly eliminates the chance for the amelogenin in EMD triggering any type of allergic reaction, even in patients with a propensity for allergies (Zetterström *et al.*, 1997).

Many believe that the cellular effects associated with EMD are due primarily to amelogenin, but again, there are conflicting studies. As previously stated, one of the more accepted affects of EMD is its ability to increase the proliferation of the cells necessary for regeneration. The idea that amelogenin is the protein responsible for this effect seems plausible since it is the main protein component in EMD. However, Viswanathan *et al.* (2003) in studying amelogenin's ability to regulate cementum-associated genes, found that the proliferative activity observed when cells were exposed to EMD was not due to

amelogenin, but some other component in the mixture. Another recent article looking at gene expression profiles of periodontal ligament cells treated with EMD, found that something (possibly amelogenin) in EMD was causing some genes (mostly inflammatory genes) to be down regulated while other genes coding for growth factors and receptors were up-regulated (Parker and Tonetti, 2004). One *in vitro* study showed that amelogenin promotes both cell attachment and spreading and, therefore, as a cellular adhesion protein, partially explains EMD's similar effects (Huang *et al.*, 2002). Another controversial theory is that amelogenin has some kind of cell signaling ability, which causes immature mesenchymal cells to change their phenotypic and maturation pathways (Veis *et al.*, 2000). This theory has received further validation in a recent *in vitro* study which found that two low molecular weight isoforms of amelogenin had distinct activities in the differentiation of the mouse tooth germ (Tompkins *et al.*, 2005). In contrast, a study using mouse molars showed that neither amelogenin nor enamelins directly participate in the formation of dentin or cementum and were not observed anywhere along the developing roots (Hu *et al.*, 2001).

In view of this information, it is clearly evident that the mechanism of action of EMD and the role of amelogenin as its major component is not well understood. More research is needed in order to fully understand its mechanism and if amelogenin is the protein component associated with the cellular effects or if it is BMP or some other minor protein.

6. THE PROBLEM – MECHANISM OF ACTION POORLY UNDERSTOOD

EMD has been proven to cause or at least enhance regeneration both histologically and clinically but what is making it work as of yet remains unknown. Amelogenin, as the major protein component of EMD, is most likely to be the component of EMD responsible

for the observed cellular effects and resulting regeneration, but this has not been definitively proven. In fact, from the studies in the literature to date, the proteins in the commercially available product known as Emdogain® have not yet been separated out and the cellular effects of the individual components compared to the known cellular effects of the parent compound. It is unknown if the individual components of EMD would stimulate the same cellular effects alone, or if there is a process requiring the orchestration of multiple elements in the EMD mixture. By answering these questions it could be shown whether or not amelogenin truly is the protein component responsible for the desired effects. Knowing this would be beneficial in several ways. If it were known that amelogenin really is the component responsible for the cellular effects, this protein could be manufactured recombinantly in its pure form in unlimited supplies, thus decreasing potential immunogenicity and increasing patient acceptance. On the other hand, if it were known that amelogenin is not responsible for the cellular effects which occur, research could move in other directions looking at more of the minor proteins such as amelin, enamelin, tuftelin or even growth factors such as BMP's or TGF- β .

The fact remains that basic science research to date has provided little evidence in understanding the mechanism by which EMD works. The most important question yet to be answered is what component of EMD is responsible for its beneficial effects.

II. INVESTIGATION PURPOSE AND AIMS

Overall Objective: To fractionate enamel matrix derivative in order to determine the extent to which proliferation, differentiation, angiogenesis, and proteolytic activities are the result of a single protein component in the mixture.

Hypothesis: Amelogenin is the protein component of enamel matrix derivative responsible for the cellular effects of differentiation and proliferation in an osteoprogenitor cell line, and proliferation and angiogenesis in human microvascular endothelial cells (HMVEC).

SPECIFIC AIM 1. To evaluate the proliferation and differentiation of C2C12 cells when stimulated by enamel matrix derivative and the potential of Noggin to inhibit the reaction.

SPECIFIC AIM 2. To evaluate the proliferation and differentiation of C2C12 cells when stimulated by amelogenin. If differentiation occurs, noggin will be used to attempt to block the reaction.

SPECIFIC AIM 3. To evaluate proliferation and angiogenesis in human microvascular endothelial cells (HMVEC) when stimulated by both EMD and amelogenin.

SPECIFIC AIM 4. To fractionate enamel matrix derivative in order to determine which component is responsible for proliferation, differentiation, angiogenesis and proteolytic activity in the various cell types. Once determined, noggin will be used on the active fractions in order to rule out the possibility of BMP being responsible for the effect.

III. MATERIALS AND METHODS

A. MATERIALS

Enamel matrix derivative (EMD) used in this study was obtained from Biora AB, Malmo, Sweden (now Straumann Biologics Division, Waltham, MA). The material, known as Emdogain[®] commercially, was supplied as a lyophilized material in 30mg vials. All material used was from the same lot number, 094093 2002-09. A stock solution was prepared by dissolving the lyophilized material in 10mM acetic acid. Freshly dissolved material was allowed to stand at 4°C for at least 1hr prior to use in order to solubilize the material. The stock solution was kept at 4°C for 7 to 10 days before discarding. Working solutions of EMD for cell culture studies were prepared by diluting the stock solution with cell culture media appropriate for the cell type studied. Control solutions were prepared similarly from 10mM acetic acid vehicle. For column separations, EMD was dissolved in 5mls of 0.05M sodium bicarbonate buffer, pH 10.8. Freshly dissolved material was allowed to stand at 4°C for at least 1hr prior to column application in order to solublize the material.

Porcine amelogenin, prepared as described by Ryu *et al.* (1999), was the generous gift of Dr. James Simmer, University of Michigan School of Dentistry (Ann Arbor, MI). Briefly, recombinant porcine amelogenin (rP172) was expressed from the pET11 expression vector in *E. coli* BL21(DE3) cells (Stratagene, La Jolla, CA, USA) and purified from *E. coli* extracts by selective precipitation in ammonium sulfate (20% saturation), followed by ion exchange chromatography, followed by separation on a C4 reversed-phase column. While EMD

(Emdogain[®]) is a mixture of many proteins, the predominant species present corresponds to the processed form of amelogenin (Fukae, 1999). Working solutions of amelogenin for cell culture studies were prepared by diluting a stock solution of 15mg/ml in 10mM acetic acid with cell culture media appropriate for the cell type studied. Control solutions were prepared similarly from 10mM acetic acid vehicle.

C2C12 cells (CRL-1772) were obtained from the American Type Culture Collection (ATCC Manassas, VA). This cell line is a subclone established by D. Yaffe and O. Saxel (1977), of a mouse myoblast cell line previously established by Blau (1985). The C2C12 cell line differentiates rapidly, forming contractile myotubes and producing characteristic muscle proteins. Treatment with bone morphogenetic protein 2 (BMP-2) causes a shift in the differentiation pathway from myoblastic to osteoblastic (Katagiri, 1994). Thus, this cell line served as an *in vitro* model cell system for evaluating the differentiation potential of EMD on osteoprogenitor cells. BMP-2 served as the control factor for stimulating differentiation of the cell line. The C2C12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% fetal bovine serum (FBS).

Human microvascular endothelial cells (HMVEC) were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). The cells used were human microvascular endothelial cells, dermal neonatal pooled. Three different lot numbers were used over the course of the project: 3F1066, 3F1489, and 4F1613. The cells were maintained in EGM2 MV medium. This medium was prepared from EBM2 medium by adding a growth factor supplement kit (see table 1) obtained from Cambrex. Cells were only used as long as they

maintained the same endothelial cell morphology evident in the parent culture, usually 4 to 5 passages.

B. METHODS

1. EMD Fractionation

EMD was fractionated using Sephadex G-100, medium (S-6147, Sigma, St. Louis, MO). A 2.5 X 100cm column was prepared and equilibrated with 0.05M sodium bicarbonate buffer, pH 10.8 (column buffer). The column flow rate was 21ml/hr. Prior to use the elution pattern of the column was determined using a mixture of blue dextran, bovine serum albumin (BSA), myoglobin, cytochrome C, and potassium dichromate. A molecular weight calibration curve was plotted from the resulting data. EMD (30mg) dissolved in 5ml of column buffer as described above was applied to the column and eluted. The eluate was collected in 20 minute (7ml) fractions. An aliquot of each fraction was assayed for protein content using the BCA protein method from Pierce Biotechnology, Inc. (Rockford, IL). The protein elution pattern was determined by plotting the protein content of each fraction aliquot versus the fraction number. A 5ml aliquot of each fraction was desalted over a PD-10 column (Amersham Biosciences, Newark, NJ) using 0.5M acetic acid and then lyophilized. The lyophilized fractions were resuspended in 0.7ml 10mM acetic acid. Prior to freezing at -20°C, the protein content of each fraction was again determined using the BCA procedure. At the time of assay the sample fractions were thawed and the protein concentration of each fraction adjusted to 50µg/ml using tissue culture medium. Some fractions did not contain detectable protein and were not tested.

2. Determination of Alkaline Phosphatase Activity

C2C12 cells were plated into 96 well plates at 2×10^4 cells / well in growth medium. After 18 hours, the medium was removed and replaced with fresh growth medium containing 5% FBS and EMD or amelogenin at concentrations of 0, 6.25, 12.5, 25, 50, and 100 μ g/ml. Fractionated EMD samples were tested at a final protein concentration of 25 μ g/ml. Fractionated EMD samples were also preincubated with 100 ng/ml noggin (R & D Systems, Minneapolis, MN) for 1 hour prior to addition to the cells. At the appropriate time (0, 2, 5, and 9 days), the medium was removed and the cell layer washed twice with phosphate buffered saline (PBS). Next, 100 μ l of a solution containing 50 μ l PBS, 16.5 μ l 1.5M 2-amino-2-methyl-1-propanol, pH 10.25, 16.5 μ l 20mM p-nitro-phenyl phosphate, and 16.5 μ l 10mM MgCl₂ was added and the cells incubated for up to 1 hour at 37°C. The reaction was stopped by the addition of 100 μ l of 1N sodium hydroxide and the absorbance read at 405nm. A standard curve was prepared from known concentrations of p-nitrophenol. The alkaline phosphatase activity was expressed as the amount of p-nitrophenol formed/well/40minutes.

3. Determination of Cell Proliferation

C2C12 cells or HMVEC cells (5×10^3 cells / well) were plated into 96 well plates using growth media appropriate for each cell type. After 18 hours, the media was removed and replaced with low serum media (5% serum C2C12, 2% serum HMVEC) containing EMD or amelogenin at concentrations of 0, 12.5, 25, 50, and 100 μ g/ml. Fractionated EMD samples were tested at a final protein concentration of 25 μ g/ml. After incubation for 72 hours at 37°C (5% CO₂: 95 % air), the medium was removed and replaced with fresh serum free culture medium containing 10% (vol : vol) WST-1 reagent (Roche, Indianapolis, IN). Incubation was continued for 2 hours at 37°C prior to determining the absorbance of the

reaction products at 450nm. This assay is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells to a soluble formazan product that is readily detectable spectrophotometrically at 450nm. The absorbance is proportional to the amount of cells in the reaction volume such that cell number can be determined by establishing a standard curve of optical density vs. known cell numbers for the cell type in question, either C2C12 or HMVEC.

4. *In Vitro* Angiogenesis Assay

The BD Biocoat[®] 96 well angiogenesis system was used (BD Discovery Labware, Bedford, MA) to determine the angiogenic activity of EMD, amelogenin, and the fractionated EMD samples *in vitro*. This system consists of a BD Falcon[™] 96-well black plate with a clear bottom that is uniformly coated with BD matrigel extracellular matrix preparation. Prior to use, the package containing the plate was removed from the -20°C freezer and allowed to thaw at a 4°C for 4 hours. Then, under a laminar flow hood, the plate was removed from the package and the flexible cover sealing the wells removed and discarded. The plate was covered with the plastic lid and placed into the cell culture incubator to allow the matrigel matrix to polymerize for 30 minutes prior to use. Twenty thousand HMVEC cells in 50µl of EBM-2 containing 5% serum but no endothelial cell growth factors were plated into the wells. An additional 50µl of medium containing EMD or amelogenin at 50µg/ml, or EMD fractions containing 50µg protein/ml, was added to the wells. Control wells contained the same amount of cells in EBM-2 medium containing 5% serum and the endothelial cell growth factors (Table 1). The plates were incubated at 37°C in humidified air containing 5% CO₂ and 95% air. At various time periods from 0 to 24 hrs, the plates were removed from the incubator and digital images obtained using brightfield

microscopy at 100X. The images were scored blindly for the extent of the angiogenic process using a scoring system developed by Chemicon International, Inc (Temecula, California) (Table 2).

5. Polyacrylamide Gel Electrophoresis

EMD and amelogenin were resolved on 15% polyacrylamide gels using the SDS-PAGE procedure described by Laemmli (1970). Aliquots of 5 or 2.5 μ g of unfractionated EMD or amelogenin, or 2 μ g of fractionated EMD were applied to the gel. Resolved bands were stained with Coomassie Brilliant Blue R-250 and photographed. For determination of enzyme activity, the gels contained denatured type I collagen. When stained, areas in which the collagen was degraded appeared clear (unstained). All electrophoresis reagents and apparatus were obtained from BioRad Laboratories (Hercules, CA).

C. DATA ANALYSIS

All data was analyzed by ANOVA with Tukey's method as the post hoc test using the GraphPad Prism statistical software program v 2.01 (Graphpad Software, Inc, San Diego, CA).

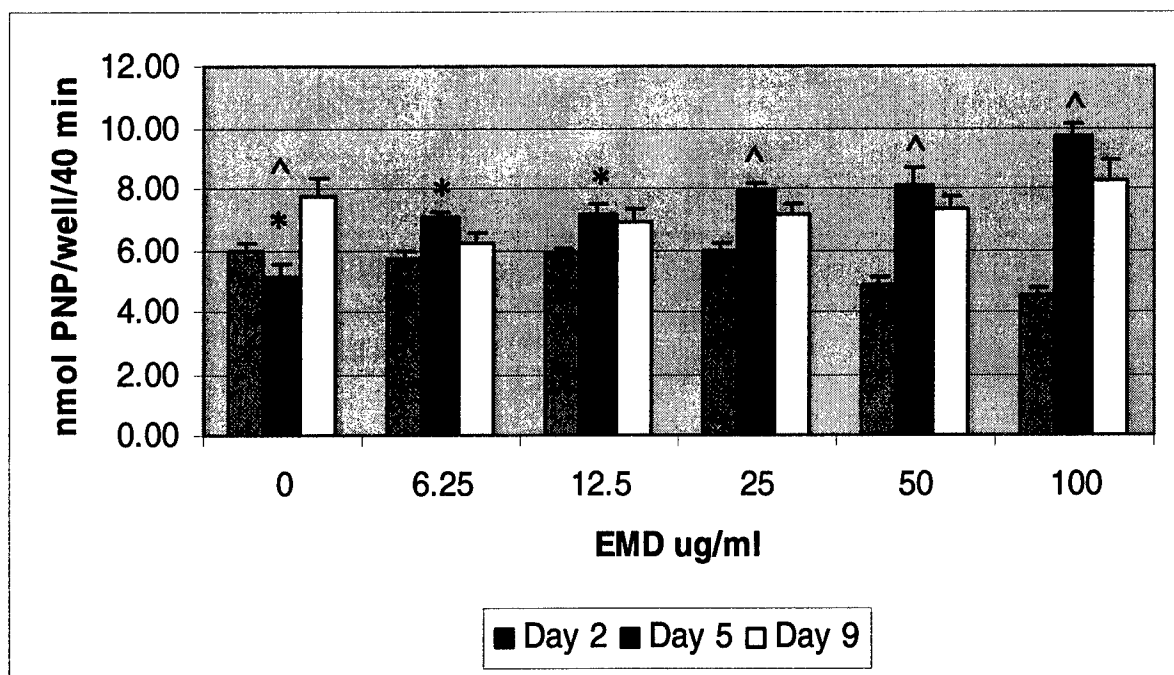
IV. RESULTS

A. EMD AND AMELOGENIN DATA

1. Alkaline Phosphatase Activity

Before separating EMD into fractions and testing for the various activities, differentiation and proliferation were evaluated using commercially obtained EMD and purified amelogenin on both C2C12 and HMVEC cell lines. Differentiation of C2C12 was evaluated on days 2, 5, and 9 by determining alkaline phosphatase activity following stimulation by EMD or amelogenin at concentrations of 6.25 μ g/ml, 12.5 μ g/ml, 25 μ g/ml, 50 μ g/ml, and 100 μ g/ml. When EMD was applied to the C2C12 cells and differentiation evaluated after 2 days, no differentiation was noted at any of the concentrations (See Fig. 1). At 50 μ g/ml, and 100 μ g/ml, differentiation appeared to be inhibited and was less than with no EMD present, although the decrease in activity was not statistically significant. At day 5, differentiation appeared to be concentration dependent, with differentiation increasing with increasing EMD concentrations. In fact, the increase in alkaline phosphatase activity was statistically significant at each concentration of EMD tested when compared to unstimulated control cells. The greatest amount of differentiation at any time period occurred at day 5 at an EMD concentration of 100 μ g/ml (See Fig. 1). At day 9, there was again no increase in alkaline phosphatase activity at any of the EMD concentrations tested compared to the unstimulated control. Thus, EMD stimulated differentiation of C2C12 cells, as indicated by alkaline phosphatase activity, in both a time and dose dependent manner on day 5 (See Fig.

Figure 1. Effect of EMD on Alkaline Phosphatase Activity of C2C12 Cells. C2C12 cells were plated into 96 well plates at 2×10^4 cells / well in growth medium. After 18 hours the medium was removed and replaced with fresh growth medium containing 5% FBS and EMD at concentrations of 0, 6.25, 12.5, 25, 50, and 100 $\mu\text{g/ml}$. At the appropriate time (0, 2, 5, and 9 days), the medium was removed and the cell layer assayed for alkaline phosphatase activity. Note that on day 2 no difference in activity was noted at any of the concentrations tested, and that at 50 $\mu\text{g/ml}$, and 100 $\mu\text{g/ml}$, activity appeared to be inhibited, although the decrease in activity was not statistically significant. At day 5, activity increased with increasing EMD concentrations, the increase being statistically significant at each concentration of EMD tested when compared to unstimulated control cells. At day 9, there was again no increase in alkaline phosphatase activity at any of the EMD concentrations tested compared to the unstimulated control. Alkaline phosphatase activity is expressed as the amount of p-nitrophenol (PNP) formed/well/40minutes. The data are the mean \pm s.e.m. (n = 8). * = $p < 0.05$, ^ = $p < 0.001$.



1). When amelogenin was applied to C2C12 cells, once again, as with EMD at day 2, differentiation was not evident and even appeared to be inhibited at all concentrations. The decreases in activity at 50 and 100 μ g/ml were significant at $p < 0.001$ (See Fig. 2). At days 5 and 9, there were no significant increases in alkaline phosphatase activity at any of the amelogenin concentrations tested, indicating that differentiation did not occur in response to stimulation by this factor (See Fig. 2).

2. Proliferation Activity

The effect of EMD and amelogenin on proliferation of C2C12 cells was examined in 5% serum containing 0, 12.5 μ g/ml, 25 μ g/ml, 50 μ g/ml, and 100 μ g/ml of the factors. Culture medium containing 10% serum and no factor served as a positive control. EMD, when applied to C2C12 cells, significantly increased cell proliferation ($p < 0.01$) only at the lowest concentration (12.5 μ g/ml) tested (See Fig. 3). By contrast, amelogenin had no effect on C2C12 proliferation at any of the concentrations tested. (See Fig. 4).

The effect of EMD and amelogenin on proliferation of HMVEC cells was examined both in 5% and 2% serum containing 0, 12.5 μ g/ml, 25 μ g/ml, 50 μ g/ml, and 100 μ g/ml of the factors. In the absence of EMD or amelogenin, HMVEC cell proliferation was significantly greater ($p < 0.001$) in medium containing 5% serum compared to medium containing 2% serum. When stimulated with EMD in medium containing 5% serum, HMVEC cell proliferation was not increased at any of the concentrations tested compared to the 5% serum control (See Fig. 5). By contrast, when stimulated with amelogenin in 5% serum containing medium, HMVEC cell proliferation was significantly increased by 6.25 μ g/ml amelogenin compared to the 5% serum control. Higher concentrations of amelogenin did not stimulate a

Figure 2. Effect of Amelogenin on Alkaline Phosphatase Activity of C2C12 Cells. C2C12 cells were plated into 96 well plates at 2×10^4 cells / well in growth medium. After 18 hours the medium was removed and replaced with fresh growth medium containing 5% FBS and amelogenin at concentrations of 0, 6.25, 12.5, 25, 50, and 100 μ g/ml. At the appropriate time (0, 2, 5, and 9 days), the medium was removed and the cell layer assayed for alkaline phosphatase activity. Note that on day 2, activity was not increased at any of the concentrations tested, and even appeared to be inhibited at all concentrations, the decreases in activity at 50 and 100 μ g/ml being significant. At days 5 and 9, there were no significant increases in alkaline phosphatase activity at any of the amelogenin concentrations tested. Alkaline phosphatase activity is expressed as the amount of p-nitrophenol (PNP) formed/well/40minutes. The data are the mean \pm s.e.m. (n = 8). * = $p < 0.001$.

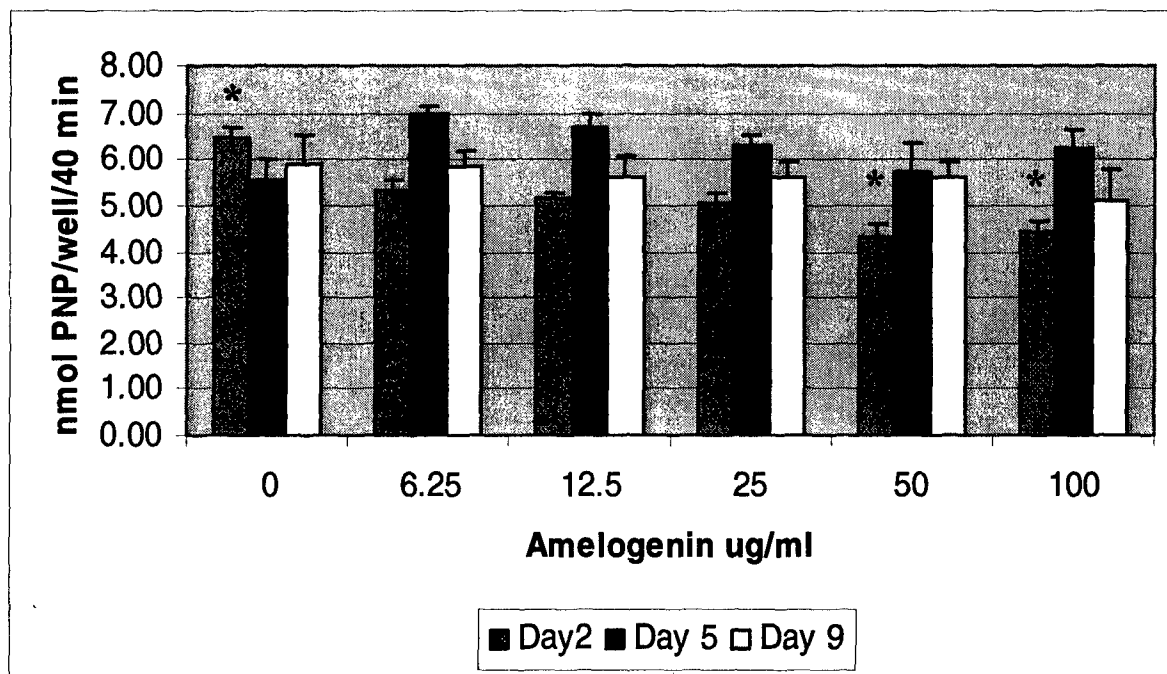


Figure 3. Effect of EMD on Proliferation of C2C12 Cells. C2C12 cells (5×10^3 cells / well) were plated into 96 well plates in growth medium. After 18 hours, the media was removed and replaced with medium containing 5% serum and EMD at concentrations of 0, 12.5, 25, 50, and 100 μ g/ml. Culture medium containing 10% serum and no EMD served as a positive control. Cell number was determined using WST-1 reagent (Roche, Indianapolis, IN). Note that EMD significantly increased cell proliferation only at the lowest concentration (12.5 μ g/ml) tested. The data are the mean \pm s.e.m. (n = 8). * = $p < 0.01$, ^ = $p < 0.001$.

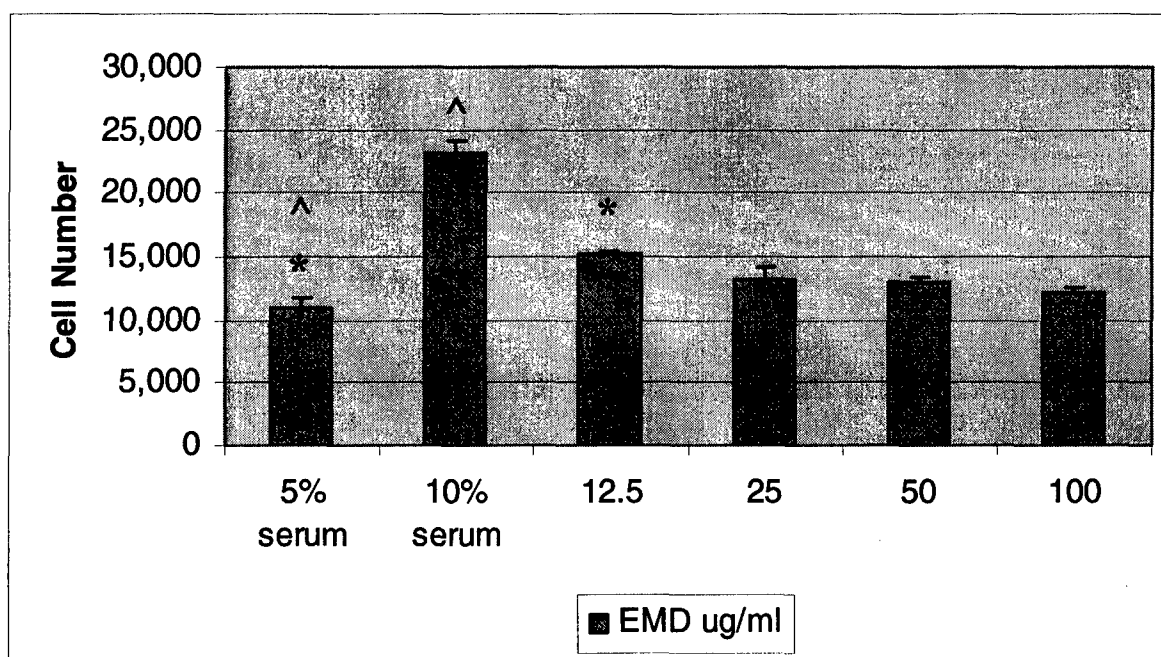


Figure 4. Effect of Amelogenin on Proliferation of C2C12 Cells. C2C12 cells (5×10^3 cells / well) were plated into 96 well plates in growth medium. After 18 hours, the media was removed and replaced with medium containing 5% serum and amelogenin at concentrations of 0, 12.5, 25, 50, and 100 μ g/ml. Culture medium containing 10% serum and no amelogenin served as a positive control. Cell number was determined using WST-1 reagent (Roche, Indianapolis, IN). Note that amelogenin had no effect on C2C12 proliferation at any of the concentrations tested. The data are the mean \pm s.e.m. (n = 8). * = $p < 0.001$.

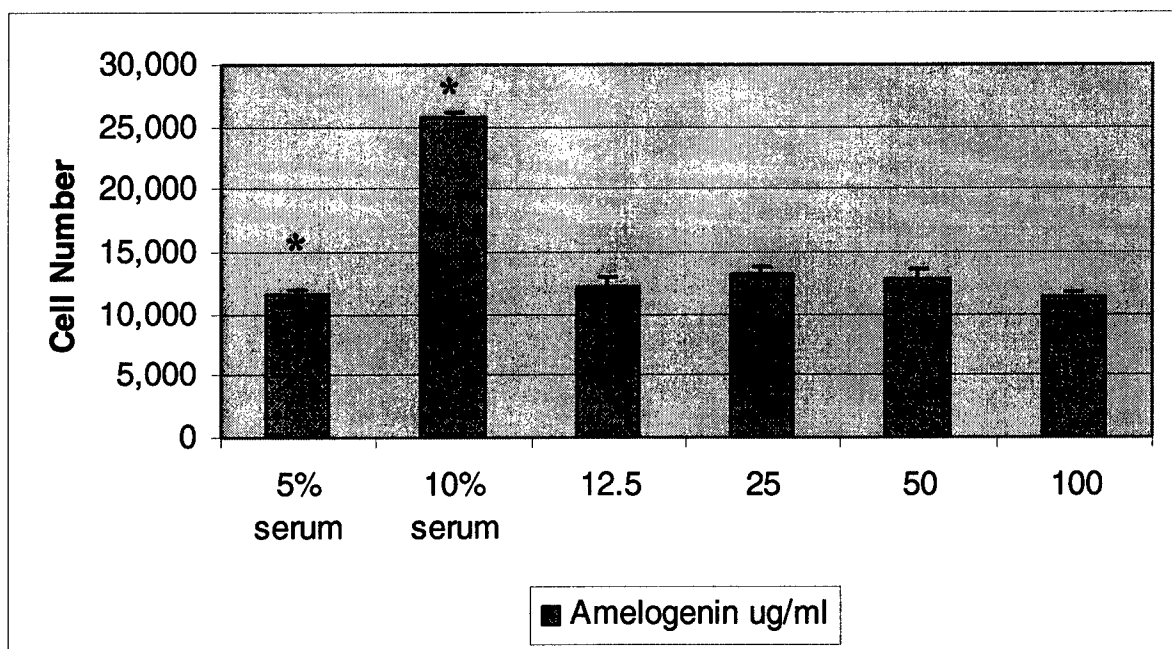
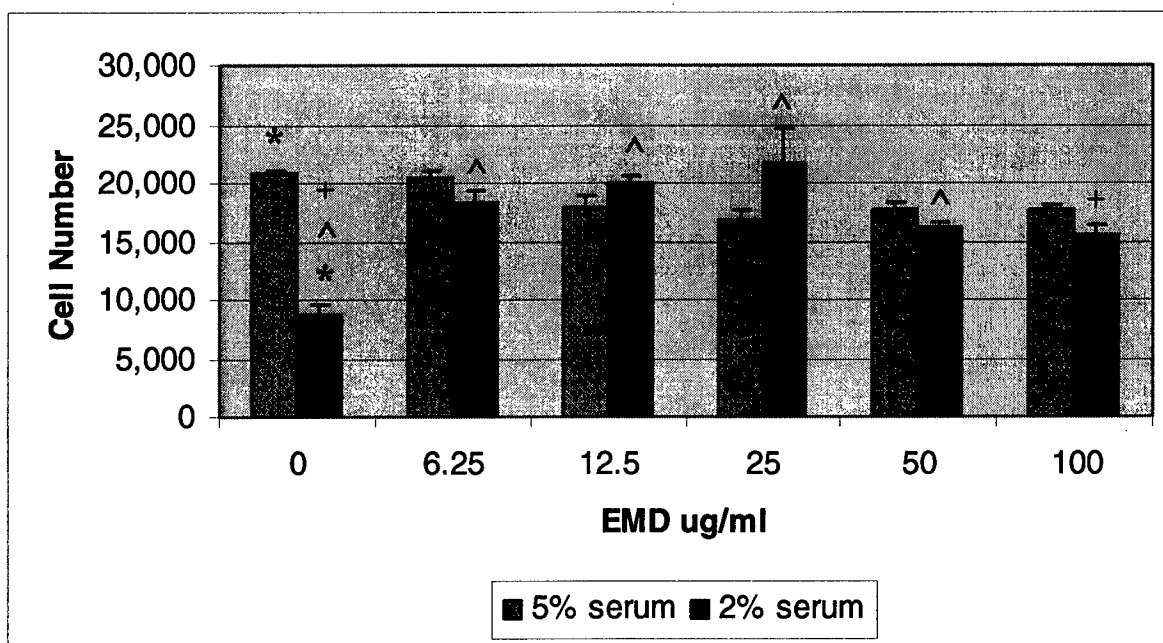


Figure 5. Effect of EMD on HMVEC Cell Proliferation. HMVEC cells (5×10^3 cells / well) were plated into 96 well plates in growth medium. After 18 hours, the media was removed and replaced with medium containing 2% serum and EMD at concentrations of 0, 12.5, 25, 50, and 100 μ g/ml. Culture medium containing 5% serum and similar concentrations of EMD served as a control. Cell number was determined using WST-1 reagent (Roche, Indianapolis, IN). Note that in the absence of EMD, HMVEC cell proliferation was significantly greater in medium containing 5% serum compared to medium alone. Note also that when stimulated with EMD in medium containing 5% serum, HMVEC cell proliferation was not increased at any of the concentrations tested compared to the 5% serum control. By contrast, EMD in medium containing 2% serum stimulated significant increases in HMVEC cell proliferation at all concentrations tested compared to the 2% serum control. Note that the greatest increase in EMD stimulated HMVEC cell proliferation occurred at 25 μ g/ml. The data are the mean \pm s.e.m. (n = 8). * and ^ = $p < 0.001$, + = $p < 0.01$.



further increase in proliferation in the 5% serum containing medium (See Fig. 6). When stimulated with either EMD or amelogenin in 2% serum containing medium, HMVEC cell proliferation was significantly increased at all concentrations tested compared to the 2% serum control. The greatest increase in EMD stimulated HMVEC cell proliferation occurred at 25 μ g/ml (See Fig. 5). By contrast, amelogenin stimulated proliferation was maximal at 6.25 μ g/ml, the lowest concentration of amelogenin tested (See Fig. 6). All concentrations tested that were greater than 6.25 μ g/ml, were not significantly different than the proliferation stimulated at this dose.

3. Angiogenesis

Angiogenesis, defined as new blood vessel formation arising from the presence of existing vasculature, was examined *in vitro* as an indicator of differentiation by microvascular cells. HMVEC cells, when cultured in EGM2 MV medium undergo rapid angiogenesis *in vitro*. This occurs because the medium contains stimulatory growth factors, particularly VEGF (See Table 1). HMVEC cells cultured in this medium served as the positive control for the angiogenesis assay. To examine the effects of EMD or amelogenin on angiogenesis, these materials were dissolved in EBM2 basal medium which is identical to EGM2 MV medium, except that it does not contain all of the growth factors listed in Table 1.

Angiogenesis was scored by taking photos of the HMVEC cells at each time period for each media type and then assigning a score of 0-5 based on the maturity of the new vascular growth. Complete and mature vessel formation, as evidenced by a complete mesh configuration of all endothelial cells, would receive a score of 5 whereas a separation only of individual cells would be scored as zero. A complete description of the scoring system can

Figure 6. Effect of Amelogenin on HMVEC Cell Proliferation. HMVEC cells (5×10^3 cells / well) were plated into 96 well plates in growth medium. After 18 hours, the media was removed and replaced with medium containing 2% serum and amelogenin at concentrations of 0, 12.5, 25, 50, and 100 μ g/ml. Culture medium containing 5% serum and similar concentrations of amelogenin served as a control. Cell number was determined using WST-1 reagent (Roche, Indianapolis, IN). Note that in the absence of amelogenin, HMVEC cell proliferation was significantly greater in medium containing 5% serum compared to medium with 2% serum. Note also that when stimulated with 6.25 μ g/ml amelogenin, containing medium with 5% serum, proliferation was significantly increased compared to the 5% serum control. Higher concentrations of amelogenin did not stimulate a further increase in proliferation in the 5% serum containing medium. When stimulated with amelogenin in medium containing 2% serum, proliferation was significantly increased at all concentrations tested compared to the 2% serum control. Note that amelogenin stimulated proliferation was not significantly different than 6.25 μ g/ml, the lowest concentration of amelogenin tested, for any concentration tested. The data are the mean \pm s.e.m. (n = 8). * and ^ = p < 0.001, + = p < 0.05.

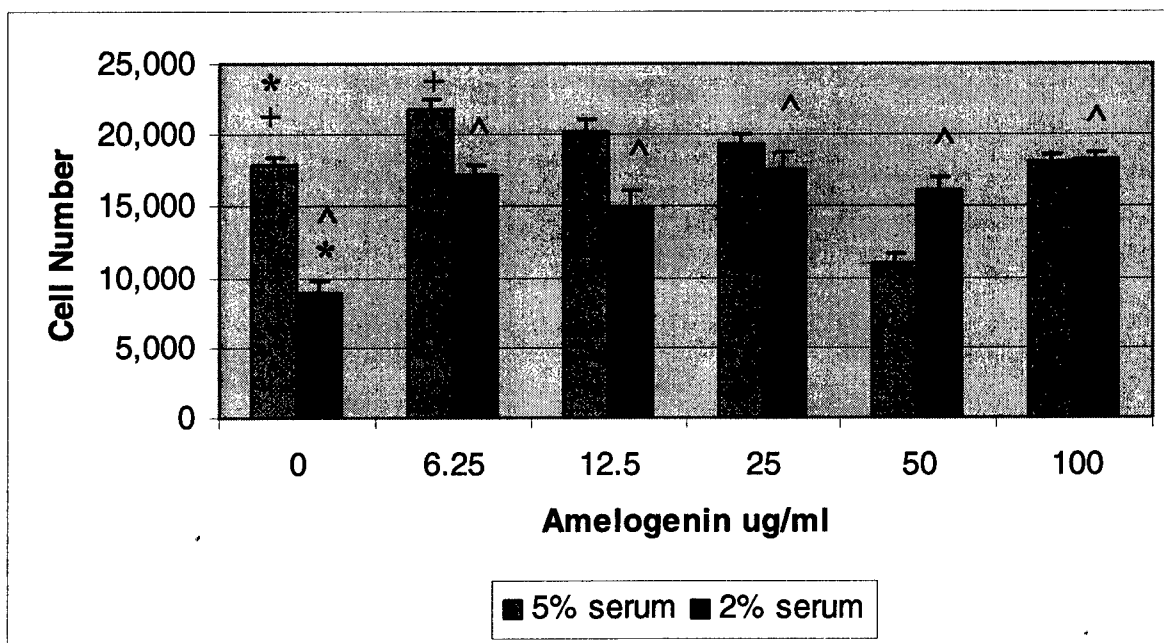


Table 1. The Media Contents of HMVEC Growth Media. Endothelial cell growth medium, EGM-2MV is prepared by adding the supplements listed in the table to endothelial cell basal medium 2 (EBM2). Both the basal medium and the supplement kit are obtained from Cambrex. EGM-2MV is the recommended medium for maintenance of the HMVEC cells supplied by the company.

GROWTH FACTOR SUPPLEMENT KIT
Gentamicin Sulfate Amphotericin-B
Ascorbic Acid
Long R Insulin-Like Growth Factor-1
Hydrocortisone
Human Fibroblast Growth Factor-B
Human Recombinant Vascular Endothelial Growth Factor
Human Recombinant Epidermal Growth Factor
Fetal Bovine Serum

be seen in table 2 and a sample, representative of each of the angiogenesis scores can be seen in figure 7.

At one hour following stimulation by EMD, there was a significant increase ($p < 0.05$) in the angiogenesis score to 0.8 ± 0.2 (mean \pm s.e.m.), indicating that the HMVEC cells had begun to migrate and align themselves. This stimulation by EMD was similar to the positive control. By contrast, the effect of amelogenin on angiogenesis at this time was not significantly different from the negative control medium that did not contain amelogenin (See Fig. 8). At four hours following stimulation by EMD, there was a significant increase ($p < 0.05$) in the angiogenesis score to 2.5 ± 0.3 (mean \pm s.e.m.), compared to the negative control (0-f), indicating that capillary tubes were now visible. Although it was not statistically significant, this stimulation by EMD was greater than the positive control (0+f). The effect of amelogenin on angiogenesis at this time was not significantly different from either the positive (0+f) or negative (0-f) control medium (See Fig. 9).

4. SDS Page Gel of EMD and Amelogenin

Before performing the column separation of EMD, an SDS-Page gel with standard molecular weight markers was run in order to compare the protein components of the EMD mixture with the purified amelogenin (See Fig. 10). Several things are apparent. EMD contains a mixture of proteins, the majority of which are relatively low molecular weight. The major EMD proteins have a molecular weight of less than 30 kDa, with the exception of one prominent band in the area of 52.2 kDa. By contrast, the major band of the amelogenin preparation occurs at a molecular weight of 28.9 kDa. Although there are a few weak bands

Table 2. *In Vitro* Angiogenesis Assay Scoring System. The progression of angiogenesis *in vitro* is scored using the criteria displayed in the table. The scoring system was developed by Chemicon International, Inc. (Temecula, California).

Pattern	Value
Individual cells, well separated	0
Cells begin to migrate and align themselves	1
Capillary tubes visible. No sprouting	2
Sprouting of new capillary tubes visible	3
Closed polygons begin to form.	4
Complex mesh like structures develop	5

Figure 7. Representative Samples of Angiogenic Scores.

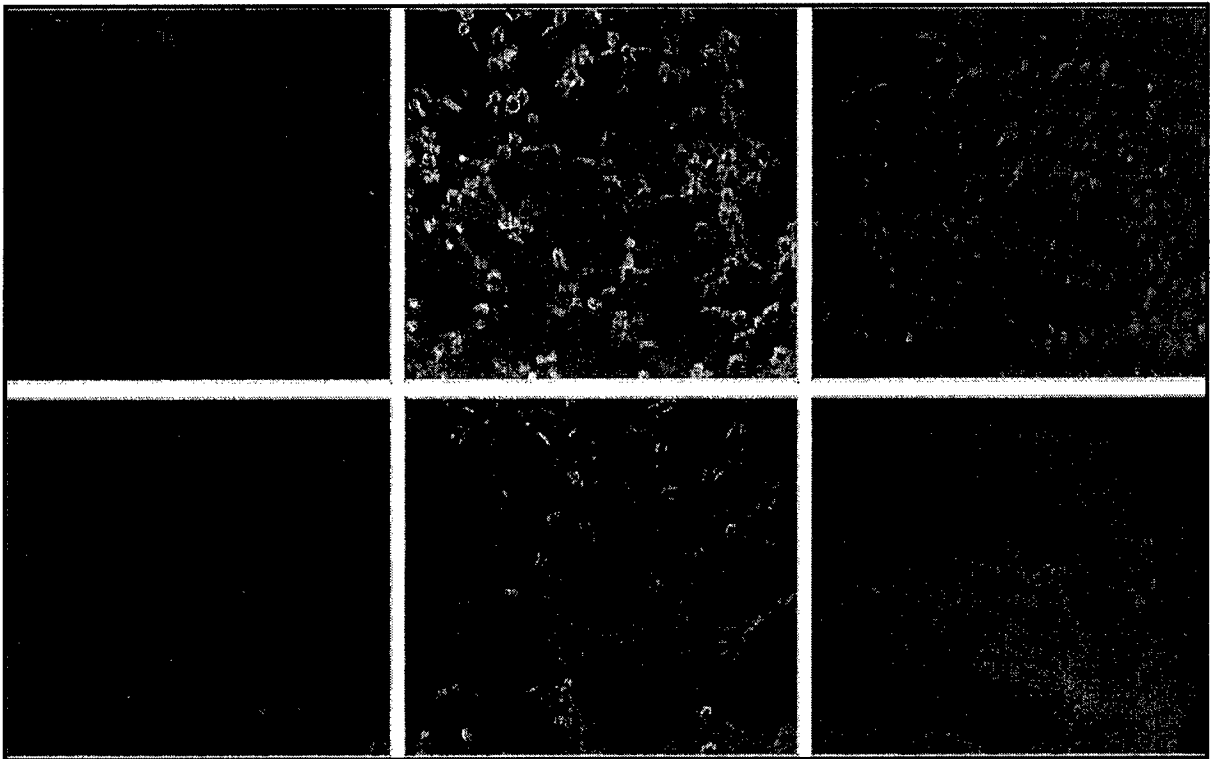


Figure 8. Effect of EMD and Amelogenin on HMVEC Cell Angiogenesis after One Hour. To examine the effects of EMD or amelogenin on angiogenesis, these materials were dissolved in EBM2 basal medium (0 - f) and applied to 20,000 HMVEC cells previously plated in the BD Biocoat® 96 well angiogenesis system. Digital images obtained using brightfield microscopy at 100X were scored blindly for the extent of the angiogenesis. Note that at one hour following stimulation by EMD (EMD - f), there was a significant increase in the angiogenesis score to 0.8 ± 0.2 indicating that the HMVEC cells had begun to migrate and align themselves. This stimulation by EMD was similar to the positive control (0 + f), which contained endothelial cell growth factors. By contrast, the effect of amelogenin (AMEL - f) on angiogenesis at this time was not significantly different from the negative control medium that did not contain amelogenin. Data are expressed as the mean \pm s.e.m. (n = 5). * and ^ = $p < 0.05$.

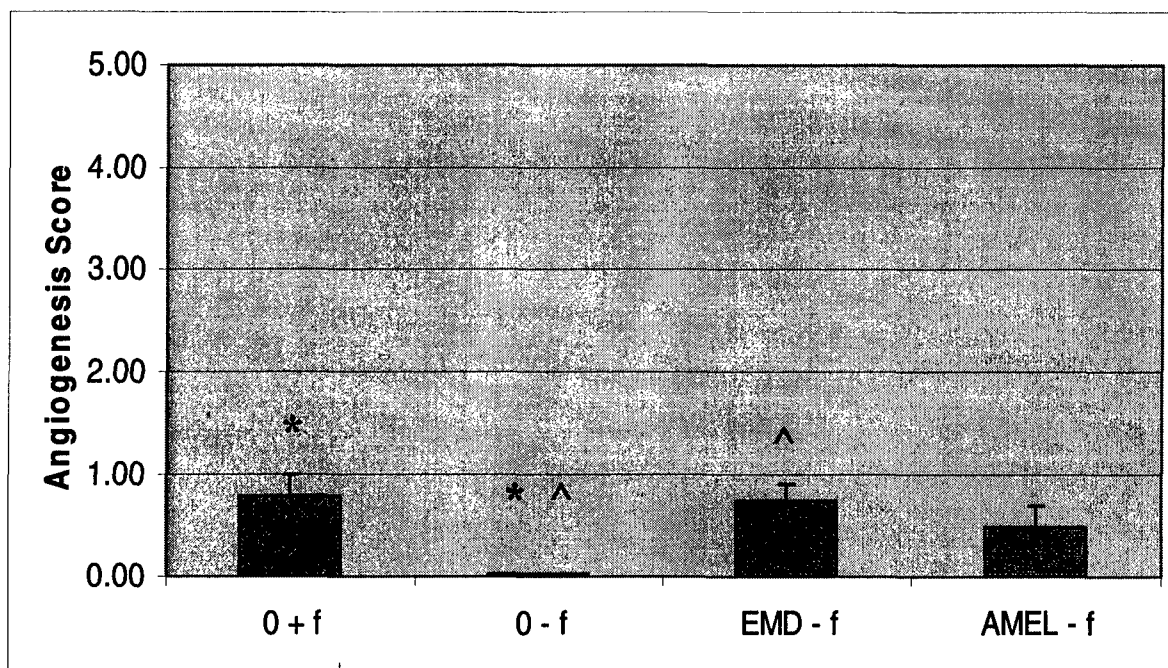


Figure 9. Effect of EMD and Amelogenin on HMVEC Cell Angiogenesis after Four Hours. To examine the effects of EMD or amelogenin on angiogenesis, these materials were dissolved in EBM2 basal medium (0 - f) and applied to 20,000 HMVEC cells previously plated in the BD Biocoat® 96 well angiogenesis system. Digital images obtained using brightfield microscopy at 100X were scored blindly for the extent of the angiogenesis. Note that at four hours following stimulation by EMD (EMD - f), there was a significant increase in the angiogenesis score to 2.5 ± 0.3 indicating that capillary tubes were now visible. Although it was not statistically significant, this stimulation by EMD was greater than the positive control (0 + f). By contrast, the effect of amelogenin (AMEL - f) on angiogenesis at this time was not significantly different from either the positive (0+f) or negative (0-f) control media. Data are expressed as the mean \pm s.e.m. (n = 5). * = $p < 0.05$.

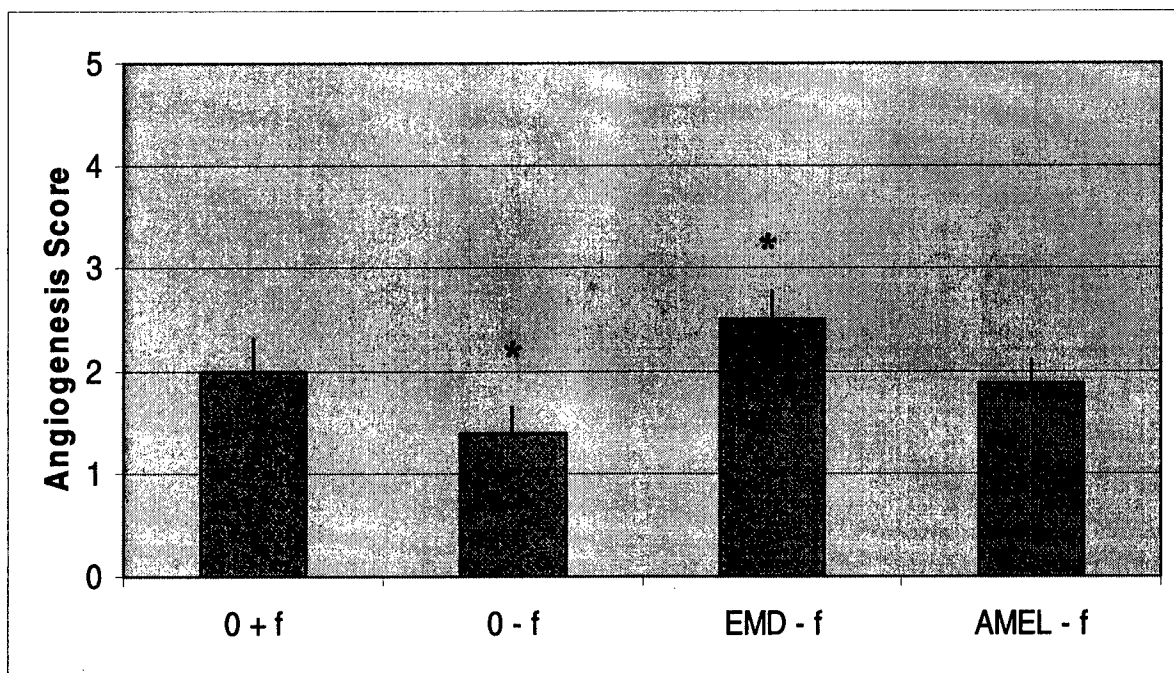
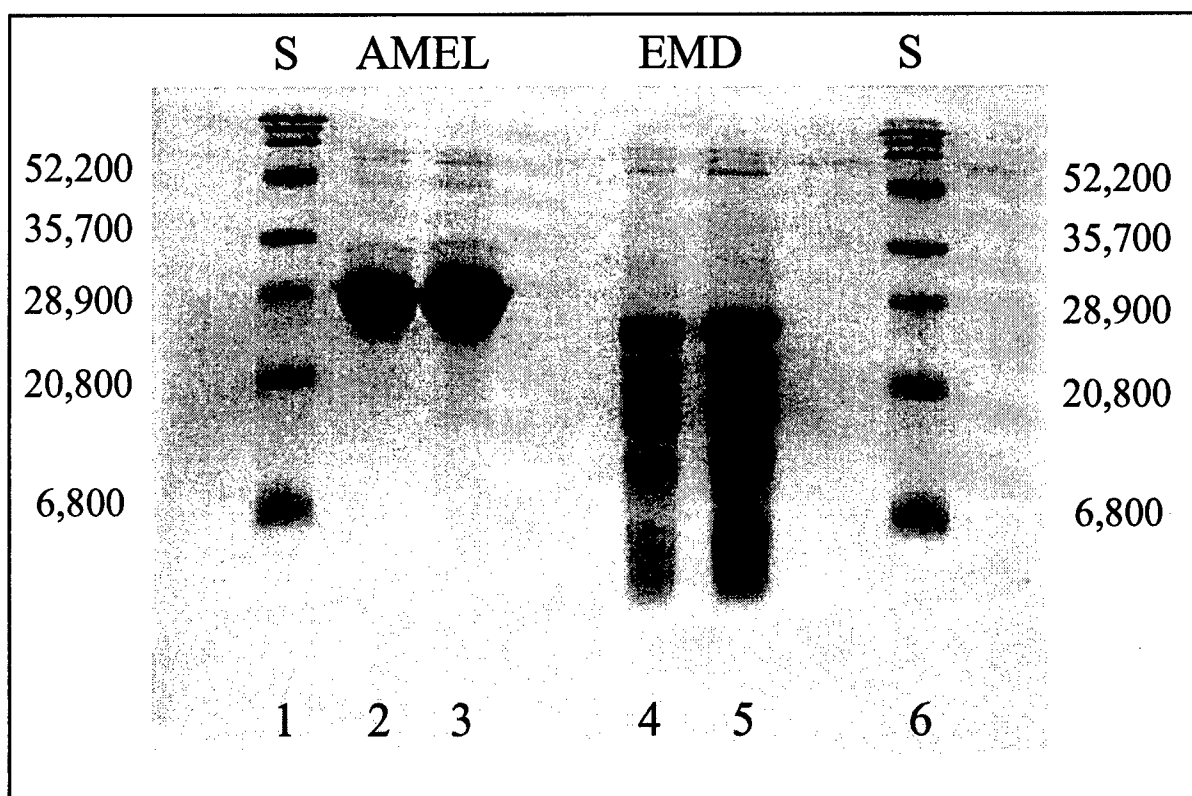


Figure 10. SDS-PAGE of Amelogenin and Enamel Matrix Derivative. EMD and amelogenin were resolved on 15% polyacrylamide gels using the SDS-PAGE procedure described by Laemmli (1970). Aliquots of 5 (lanes 3, 5) or 2.5 μ g (lanes 2, 4) of EMD or amelogenin were applied to the gel. Standard molecular weight markers were run in lanes 1 and 6. Resolved bands were stained with Coomassie Brilliant Blue R-250 and photographed. Note that EMD contains a mixture of proteins, the majority of which are relatively low molecular weight, less than 30 kDa, with the exception of one prominent band in the area of 52.2 kDa. By contrast, the major band of the amelogenin preparation occurs at a molecular weight of 28.9 kDa. Although there are a few weak bands present at higher molecular weights greater than 50 kDa, there are no protein bands present at molecular weights below 28.9 kDa.



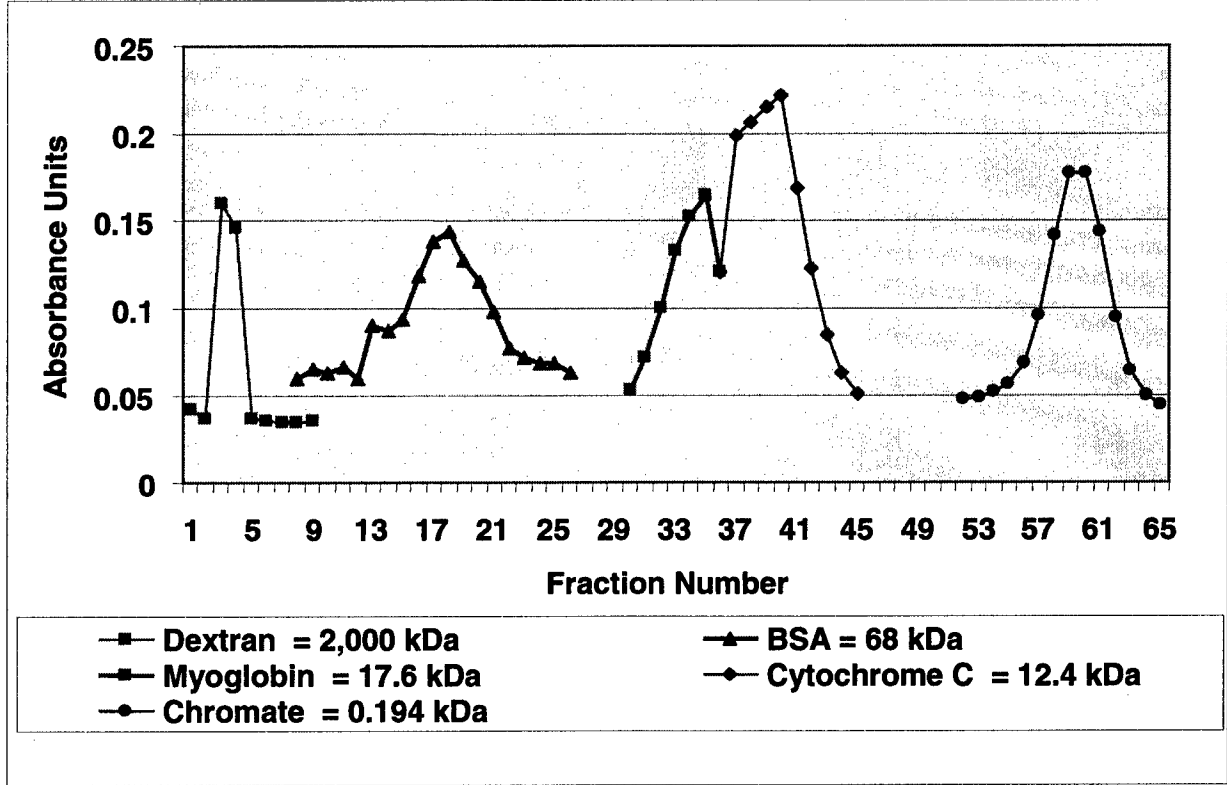
present at higher molecular weights greater than 50 kDa, there are no protein bands present at molecular weights below 28.9 kDa (See Fig. 10). As a result of this data, a Sephadex G-100 column was employed to fractionate the EMD protein mixture. This column separates proteins that are in the range of 10 to 100 kDa from one another.

B. SEPHADEX G-100 COLUMN CALIBRATION

Before applying EMD to the column to separate the various proteins in the mixture, the Sephadex G-100 column was calibrated using markers of known molecular weights. Two things were accomplished by doing this, one: it assured that the column was indeed functioning properly, and two: it allowed determination of when/where molecules of known molecular weights elute from the column. The following markers with their accompanying molecular weights were used, dextran (2000 kDa), bovine serum albumin (BSA 68 kDa), myoglobin (17.6 kDa), cytochrome C (12.4 kDa), and potassium chromate, which is not a protein but a salt with a molecular weight of 94.

By using the high molecular weight carbohydrate, dextran, a carbohydrate that is greater than approximately 100 kDa, the void volume was determined. This represents the volume after which all molecules greater than 100 kDa elute or exit the column. Due to the nature of the Sephadex gel (G-100), all molecules greater than 100 kDa, run directly through without being impeded and are the first proteins or molecules to exit the column. The dextran exited the column in fraction numbers 2-4 (See Fig. 11). Note that all of the marker does not elute in a single 7ml fraction, but rather multiple fractions, thereby establishing a peak when fraction numbers plotted on the X axis are correlated with absorbance units on the

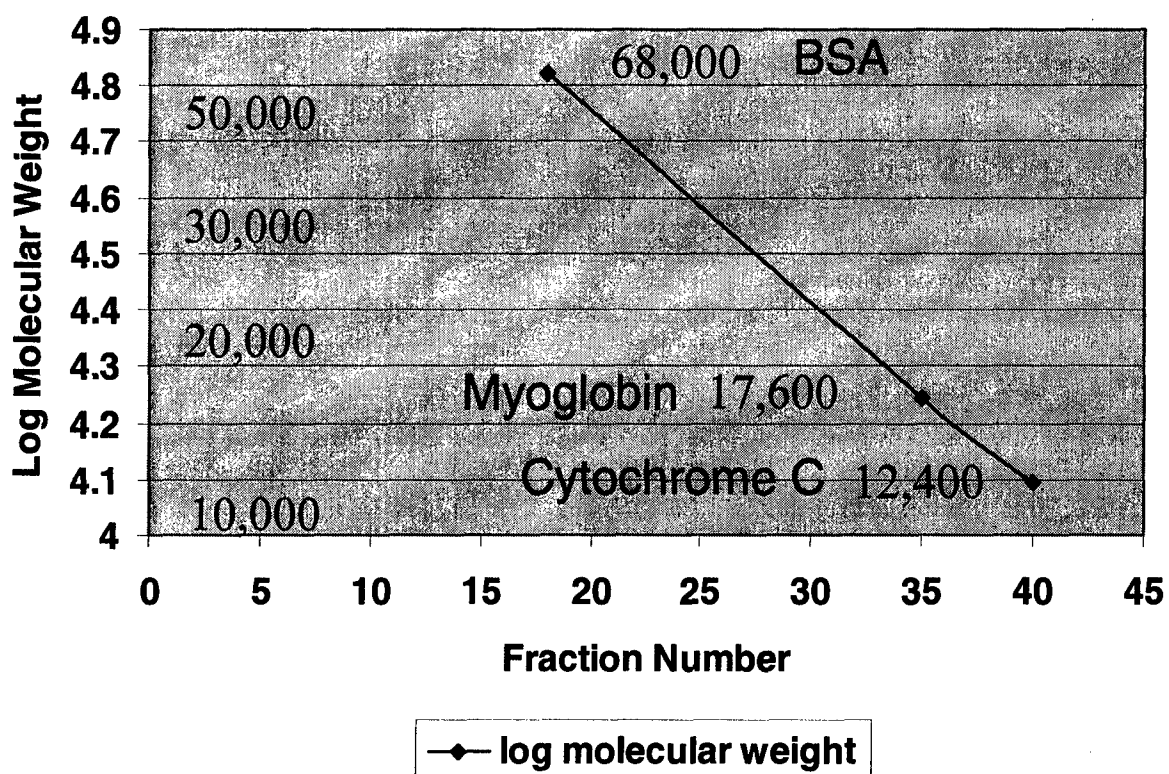
Figure 11. Sephadex G-100 Column Calibration Molecular Weight Markers. EMD was fractionated using 2.5 X 100cm column of Sephadex G-100 equilibrated with 0.05M sodium bicarbonate buffer, pH 10.8 (column buffer). The column flow rate was 21ml/hr. Note the elution positions of the markers: blue dextran, bovine serum albumin (BSA), myoglobin, cytochrome C, and potassium dichromate.



Y axis. It is important to note that the height of the peak is not correlated to molecular weight, but to the amount of actual protein in the individual fraction.

Potassium chromate was used to determine when the last molecules elute from the column. This low molecular weight salt is retarded more than any protein as it passes through the G-100 column. The complete elution of this molecule from the column signals that everything applied to the column for separation has exited in prior fractions and that the column is now clear of protein and small molecular weight molecules. Potassium chromate eluted from the column in fraction numbers 54-64 (See Fig. 11). Proteins with molecular weights between those of potassium chromate and 100 kDa, the upper limit of the columns ability to separate protein, elute in different fraction numbers depending on their molecular weights. Proteins with higher molecular weights elute first while smaller proteins elute in later fraction numbers. This is apparent from the elution positions of BSA, myoglobin, and cytochrome C, which elute in fraction numbers, 13-23, 30-36, and 37-44 respectively. Figure eleven displays the established protein elution profile of the column used in the present study. By plotting the log of the known molecular weights of BSA, myoglobin, and cytochrome C, versus the fraction number corresponding to the peak in which they eluted from the column, a standard curve relating fraction number to molecular weight was established (See Fig. 12). This calibration curve was used to estimate the molecular weights of unknown protein(s) that eluted from the column and were associated with distinct biological activities.

Figure 12. Molecular Weight Standard Curve. A molecular weight calibration curve of the log of the molecular weight versus the elution fraction number was plotted from the elution positions of the marker proteins, bovine serum albumin (BSA), myoglobin, and cytochrome C.



C. EMD PROTEIN SEPARATION

Thirty milligrams of EMD was fractionated during each column run. The protein concentration of each fraction was determined spectrophotometrically and plotted as a function of fraction number in order to establish the protein separation profile for EMD (See Fig. 13). The proteins in EMD separate into 3 distinct peaks. The first peak represents high molecular weight proteins associated with the void volume that go directly through the column without being retarded. The second protein peak elutes between the BSA and myoglobin calibration proteins, while the 3rd protein peak elutes slightly after the cytochrome c marker. A comparison of the major protein peaks in the EMD protein separation profile with the proteins of known molecular weights is displayed in figure 14. The standard curve of the proteins of known molecular weights (See Fig. 12) was used to approximate the molecular weights of each of the major protein peaks in the EMD protein separation profile (See Fig. 15). The second protein peak is approximately 50 kDa or 50000 MW while the 3rd peak was extrapolated to be about 10 kDa, or 10000 MW. The first peak, associated with the void volume, contains those proteins that are 100 kDa or 100000 MW or greater. It is interesting to note, that proteins known to be a part of the EMD mixture such as amelogenin, amelins, and tuftelin, if pure, would fall along the second protein peak of the chromatogram while enamelins would fall within the void volume peak (See Fig. 15).

D. FRACTIONATED ALKALINE PHOSPHATASE ACTIVITY

The amount of protein in each fraction was determined using the BCA protein assay. Cell culture media was used to adjust the protein concentration in each fraction to 50µg/ml prior to determining the biological activity of the fractions. Biological activities examined

Figure 13. EMD Protein Separation Profile. EMD was fractionated over a 2.5 X 100cm Sephadex G-100 column equilibrated with 0.05M sodium bicarbonate buffer, pH 10.8 (column buffer). The column flow rate was 21ml/hr. EMD (30mg) dissolved in 5ml of column buffer was applied to the column and eluate collected in 20 minute (7ml) fractions. An aliquot of each fraction was assayed for protein content using the BCA protein assay and the protein elution pattern determined by plotting the protein content of each fraction versus the fraction number.

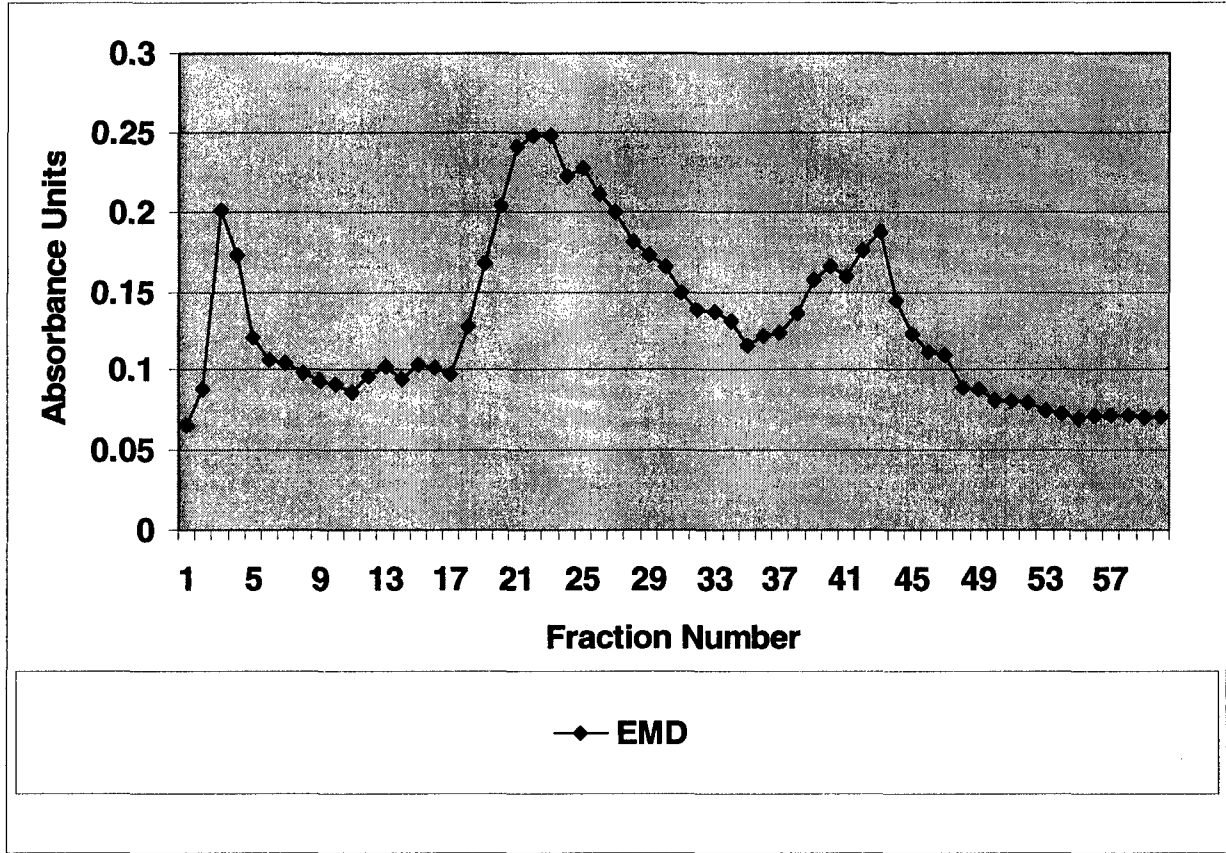


Figure 14. Fractionation of EMD Over Sephadex G-100 Column with Molecular Weight Markers. The figure displays the elution position of the marker proteins superimposed over the elution profile of fractionated EMD.

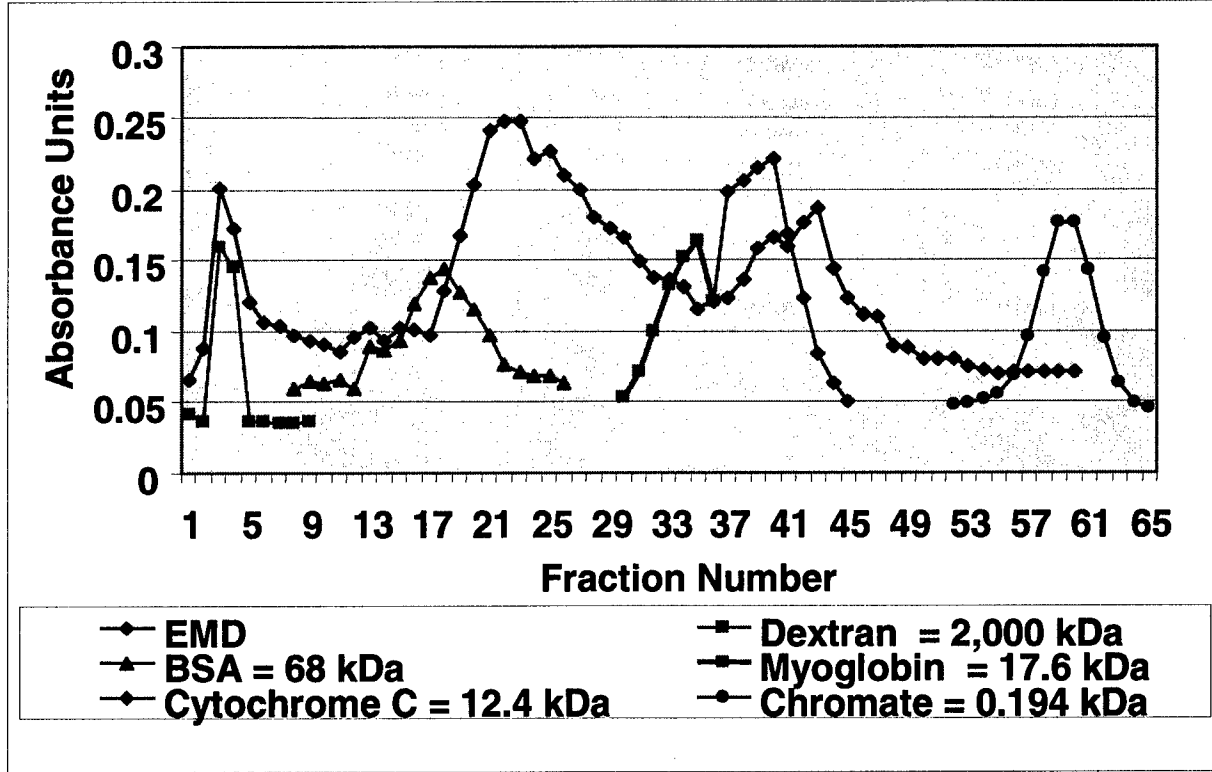
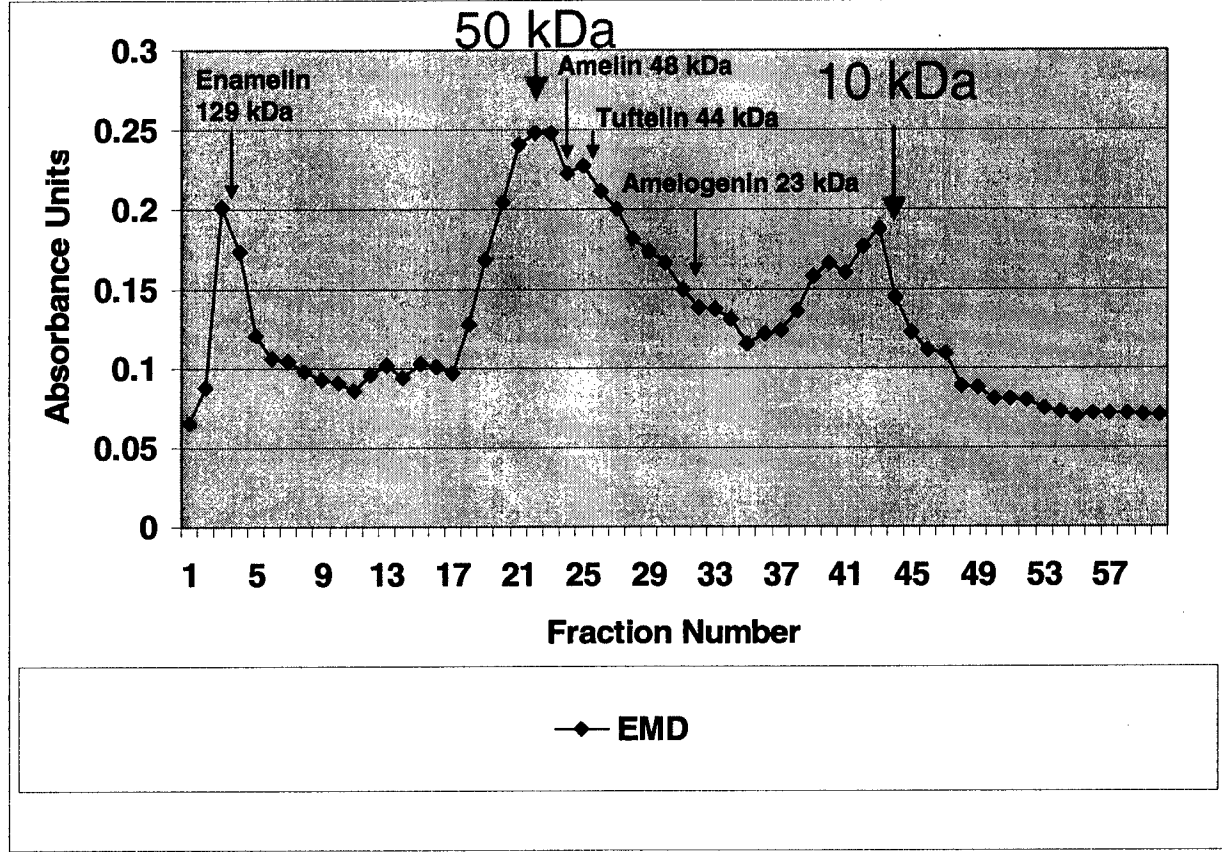


Figure 15. Molecular Weight of EMD Protein Peaks. The figure displays the elution pattern of fractionated EMD. The molecular weights of the major peaks are indicated by the arrows at 50 kDa and 10 kDa. Also, displayed are the expected elution positions of proteins known to be present in EMD based on their molecular weights.



included differentiation, proliferation, and angiogenesis, as well as collagenolytic activity. The ability of C2C12 cells to differentiate into osteoprogenitor, or bone-forming cells is well known and is characterized by an increase in the expression of alkaline phosphatase activity.

When the alkaline phosphatase activity of each fraction was determined and superimposed on the EMD protein separation profile (See Fig. 16), it was evident that the strongest alkaline phosphatase activity occurred in fractions 12-17, which does not correspond to any of the major protein peaks found in EMD. In fact, there appears to be little activity in the fractions containing the major EMD protein peaks. When the fractions were preincubated with 100 ng/ml of noggin, a decoy receptor known to bind to BMP 2, 4, and 7, thus blocking their activity, the alkaline phosphatase activity was decreased, especially in the area where the highest activity occurred prior to noggin treatment (See Fig. 17). These data are consistent with the suggestion that the osteoinductive activity associated with EMD is not related to amelogenin, but more likely with a member of the BMP family of proteins, and that only a small amount of protein accounts for this activity.

E. FRACTIONATED PROLIFERATION ACTIVITY

The proliferation of C2C12 and human microvascular endothelial cells (HMVEC) was determined and superimposed on the protein separation profile for EMD. Unlike alkaline phosphatase activity, the proliferative activity for C2C12 cells (See Fig. 18) and HMVEC cells (See Fig. 19) mirrored the EMD protein separation profile, with the greatest proliferative activity occurring in the fractions coincident with the major protein peaks. This data is consistent with the suggestion that the proliferative activity for both the osteoprogenitor and human microvascular endothelial cells is associated with the major

Figure 16. Alkaline Phosphatase Activity of EMD Fractions on C2C12 Cells. The protein concentration of each fraction was adjusted to 50µg/ml using tissue culture medium. Some fractions did not contain detectable protein and were not tested. Note that when the alkaline phosphatase activity of each fraction was determined and superimposed on the EMD protein separation profile, the strongest activity occurred in fractions 12-17, which does not correspond to any of the major EMD protein peaks.

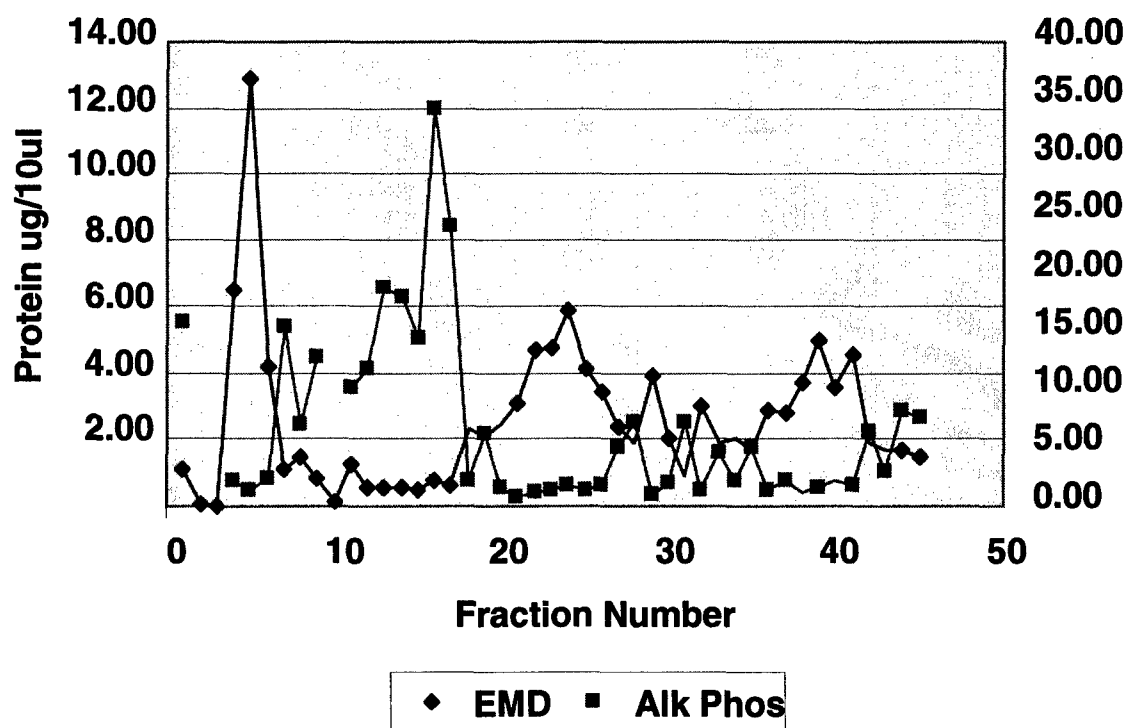


Figure 17. Noggin Inhibition of Alkaline Phosphatase Activity of EMD Fractions on C2C12 Cells. The protein concentration of each fraction was adjusted to 50µg/ml using tissue culture medium. Some fractions did not contain detectable protein and were not tested. Prior to application on the cells, the fractions were preincubated for 1 hour with 100ng/ml noggin, a decoy receptor known to bind to BMP 2, 4, and 7, and block their activity. Note that alkaline phosphatase activity was decreased, especially in the area where the highest activity occurred prior to noggin treatment. These data are consistent with the suggestion that the osteoinductive activity associated with EMD is not related to amelogenin, but more likely with a member of the BMP family of proteins.

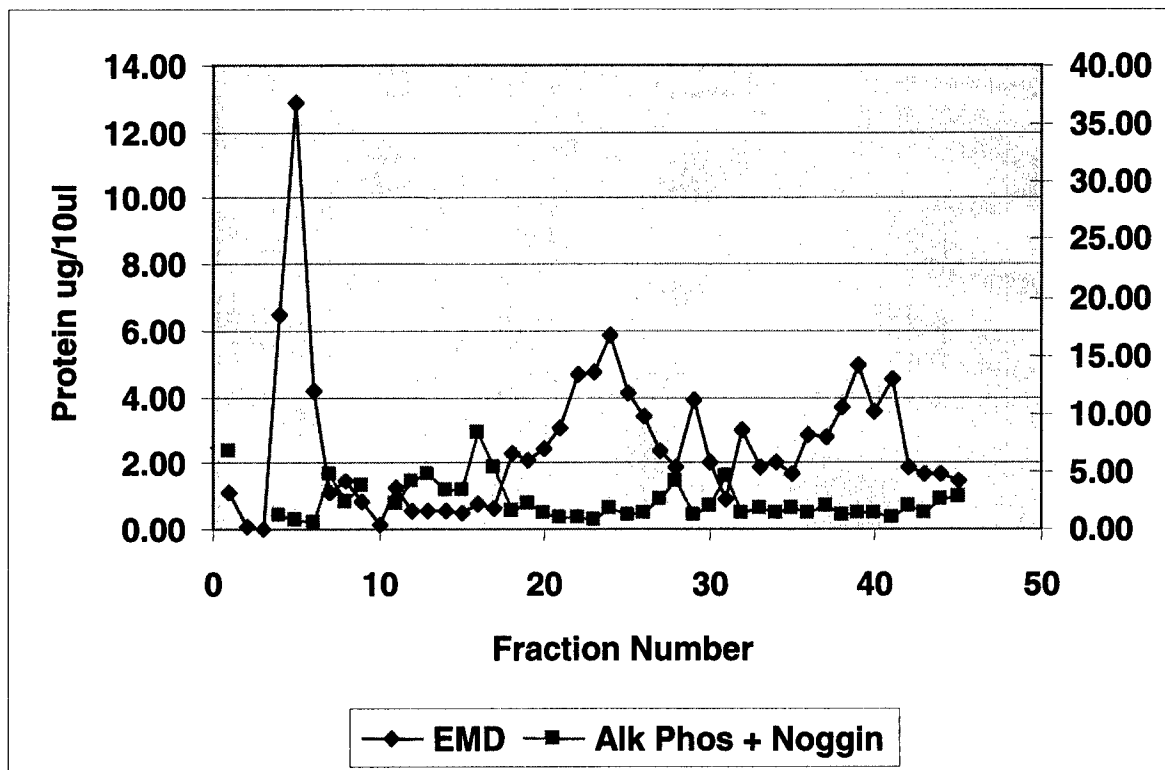


Figure 18. Proliferation Activity of EMD Fractions on C2C12 Cells. Proliferation of C2C12 cells was determined and superimposed on the protein separation profile for EMD. Note that the proliferative activity for C2C12 cells mirrored the EMD protein separation profile, with the greatest proliferative activity occurring in the fractions coincident with the major protein peaks. The protein concentration of each fraction was adjusted to 50 μ g/ml using tissue culture medium prior to assay. Some fractions did not contain detectable protein and were not tested.

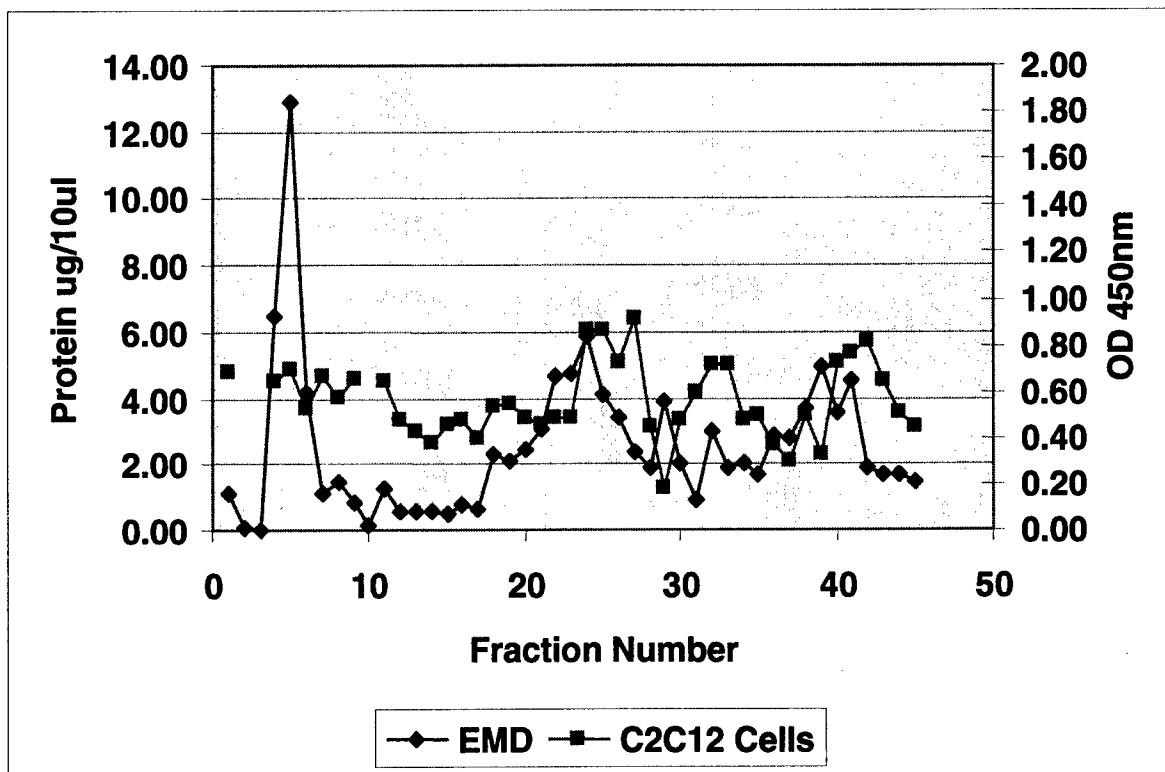
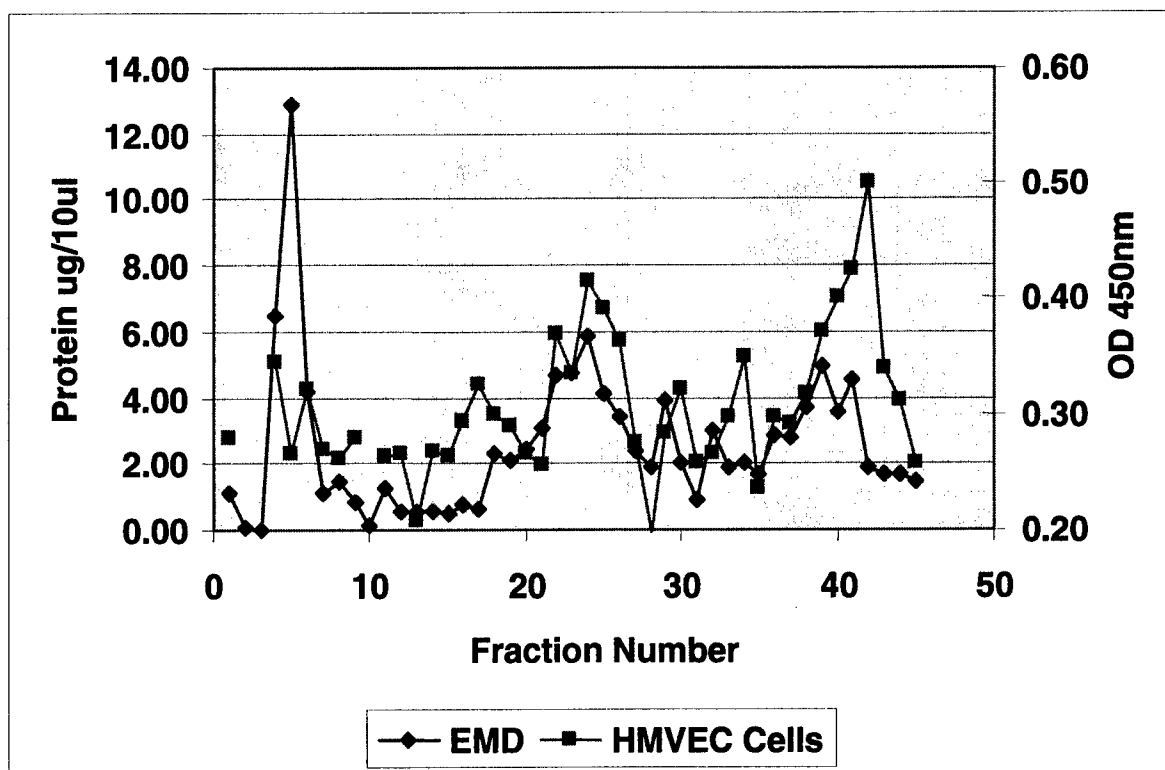


Figure 19. Proliferation Activity of EMD Fractions on HMVEC Cells. Proliferation of human microvascular endothelial cells (HMVEC) was determined and superimposed on the protein separation profile for EMD. Note that the proliferative activity for HMVEC cells mirrored the EMD protein separation profile, with the greatest proliferative activity occurring in the fractions coincident with the major protein peaks, particularly the 10kDa peak. The protein concentration of each fraction was adjusted to 50 μ g/ml using tissue culture medium. Some fractions did not contain detectable protein and were not tested.



protein components of the EMD mixture, but is distinct from the proteins associated with the induction of alkaline phosphatase activity.

F. FRACTIONATED ANGIOGENIC ACTIVITY

EMD was fractionated and applied to the HMVEC cells at a concentration of 25µg/ml, and angiogenesis scored at 1 and 4 hours. When the scores were plotted against fraction number and superimposed on the EMD protein separation profile, angiogenic activity at one hour was present in the same fractions as the 2nd and 3rd EMD protein peaks (See Fig. 20). The angiogenesis score of 1.0 for each of the peaks may initially appear insignificant, but when compared to the angiogenic activity of the unfractionated EMD at 1 hour (angiogenesis score of 0.8), it is evident that there is a slight increase in angiogenic activity in the fractions. It is also interesting to note that at one hour, there is no activity associated with the void volume peak. At 4 hours an angiogenesis score of 3.0 corresponded to the void volume peak (See Fig. 21). Furthermore, even though the angiogenic activity peaks at 4 hours, once again corresponding to the 2nd and 3rd EMD peaks, there were several important differences. The angiogenic activity corresponding to the second peak was increased significantly (angiogenesis score of 4.0), signifying almost complete vascular maturity and organization, while activity associated with the 3rd protein peak, although present, remained the same as at 1 hour (angiogenesis score of one). These data are consistent with the interpretation that angiogenesis, like proliferation, is associated with the major protein peaks found in EMD, particularly peak 2 that likely contains amelogenin. The fact that purified amelogenin exhibited little if any angiogenic activity (See Figures 8 and 9),

Figure 20. Angiogenic Activity of EMD Fractions on HMVEC Cells at 1 Hour. EMD was fractionated, applied to the HMVEC cells at a concentration of 25µg/ml, angiogenesis scored and plotted against fraction number, and superimposed on the EMD protein separation profile. Note that angiogenic activity at one hour was coincident with the 2nd and 3rd EMD protein peaks. Note also that at one hour, there is no activity associated with the void volume peak.

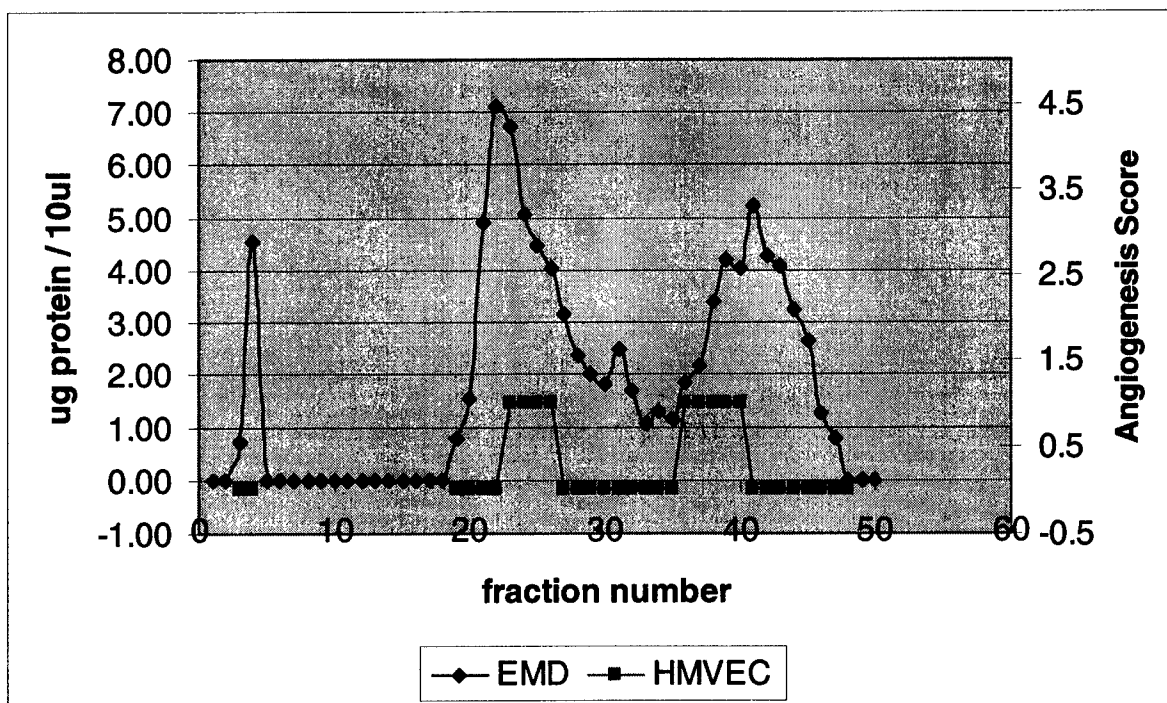
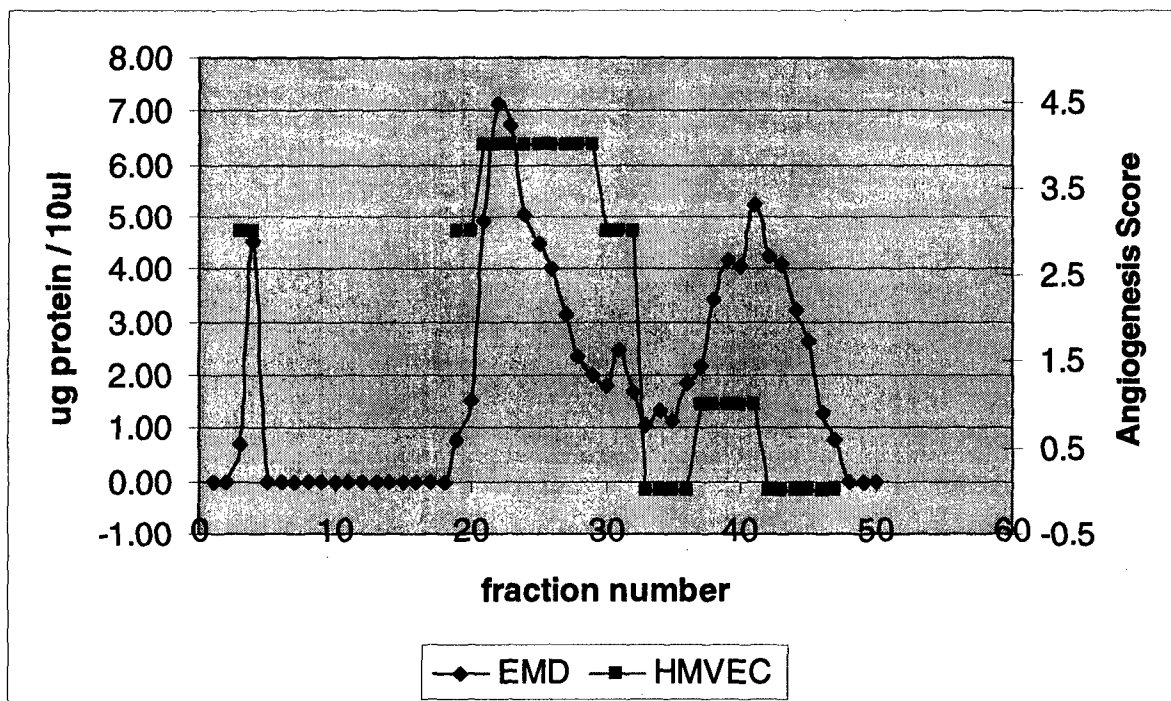


Figure 21. Angiogenic Activity of EMD Fractions on HMVEC Cells at 4 Hours. EMD was fractionated, applied to the HMVEC cells at a concentration of 25µg/ml, angiogenesis scored and plotted against fraction number, and superimposed on the EMD protein separation profile. Note that angiogenic activity at four hours was coincident with the 2nd and 3rd EMD protein peaks. Note also that an angiogenesis score of 3.0 is associated with the void volume peak. Note that the angiogenic activity corresponding to the second peak was increased over that present at one hour, while activity associated with the 3rd protein peak, although present, remained the same as at 1 hour (See Fig. 20).



suggests that the angiogenic activity present in this region may be associated with proteins other than amelogenin.

G. FRACTIONATED COLLAGENOLYTIC ACTIVITY

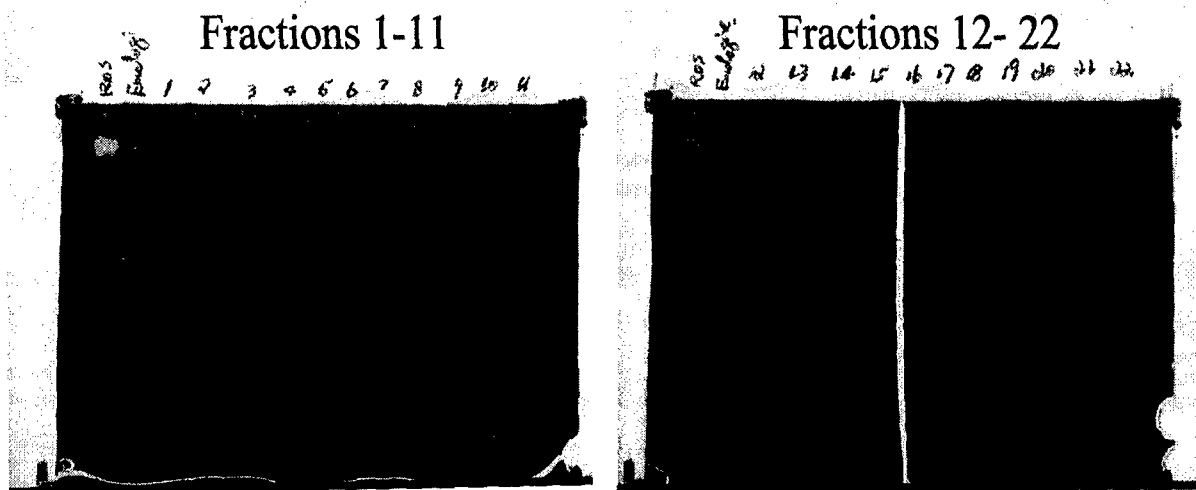
To our knowledge, the association of enzymatic or collagenolytic activity with EMD has not been previously reported in the literature. Therefore, the ability of the EMD fractions to degrade denatured type I collagen was evaluated by utilizing polyacrylamide gel zymograms. In each of the gels, lane 1 was a standard preparation derived from the rat osteosarcoma cell line ROS 17/2.8, and used to verify that the gels functioned properly. Lane two contained unfractionated EMD. After electrophoreses, collagenolytic activity was evident in two areas, fractions 3-8 and 17-21 (See Fig. 22a). Thus, the collagenolytic activity of EMD was associated with proteins in peak 1, as well as with proteins eluting in the same molecular weight region as BSA (See Fig. 22b), prior to the elution of amelogenin. There was no further collagenolytic activity apparent in any of the later eluting fractions. These data are the first to demonstrate that EMD contains enzymes that are capable of degrading type I collagen, and suggest that a more detailed analysis of the protein degrading activity of EMD is warranted.

H. SDS PAGE GELS OF FRACTIONATED EMD

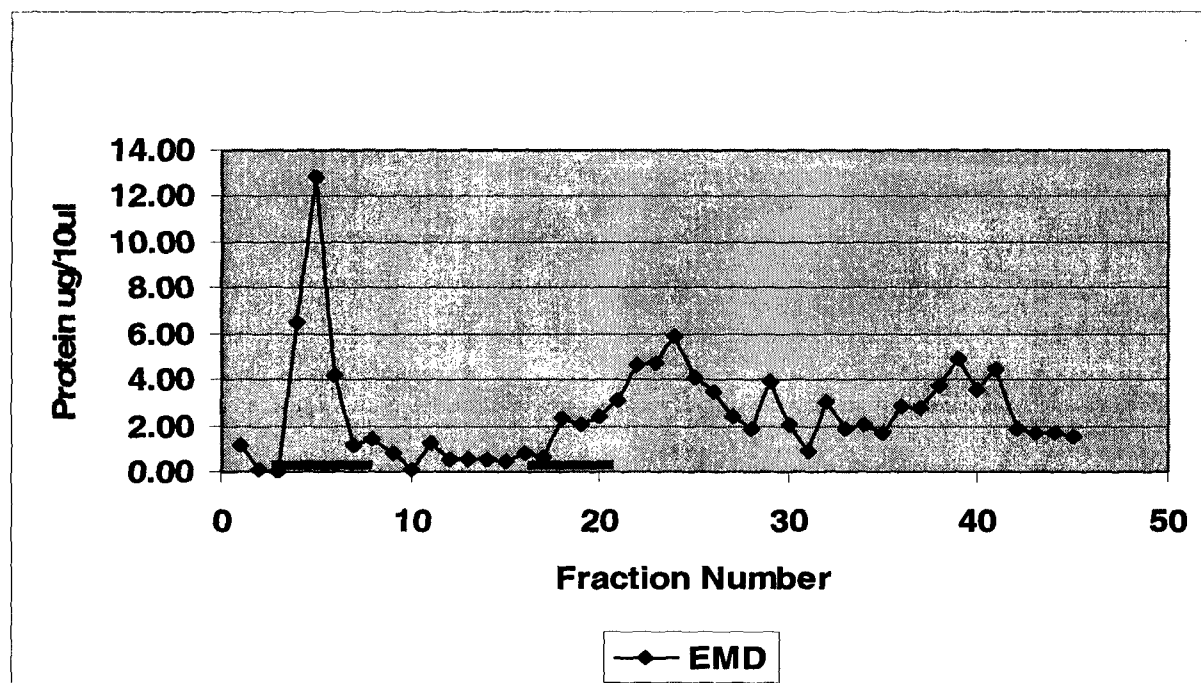
Finally, for comparison purposes with unfractionated EMD, each of the fractions was run on an SDS page gel with molecular weight markers. On the gel, there were no protein bands evident in any of the fractions from 0-18. In fraction nineteen, a very strong band

Figure 22. Zymograms of EMD Column Fractions. EMD fractions were resolved on 15% polyacrylamide gels containing denatured type I collagen. Lane 1 was a standard from the rat osteosarcoma cell line (ROS) used to verify that the gels functioned properly, while lane two contained unfractionated EMD. The resulting gels were stained with Coomassie Brilliant Blue R-250 and photographed. Note that areas in which the collagen was degraded appear clear (unstained). Note that collagenolytic activity was evident in two areas, fractions 3-8 and 17-21(Fig. 22a). Thus, the collagenolytic activity of EMD was associated with proteins in peak 1 (Fig. 22b), as well as with proteins eluting at the same molecular weight region as BSA (68 kDa), prior to the elution position of amelogenin. There was no further collagenolytic activity apparent in any of the later eluting fractions.

22a



22b

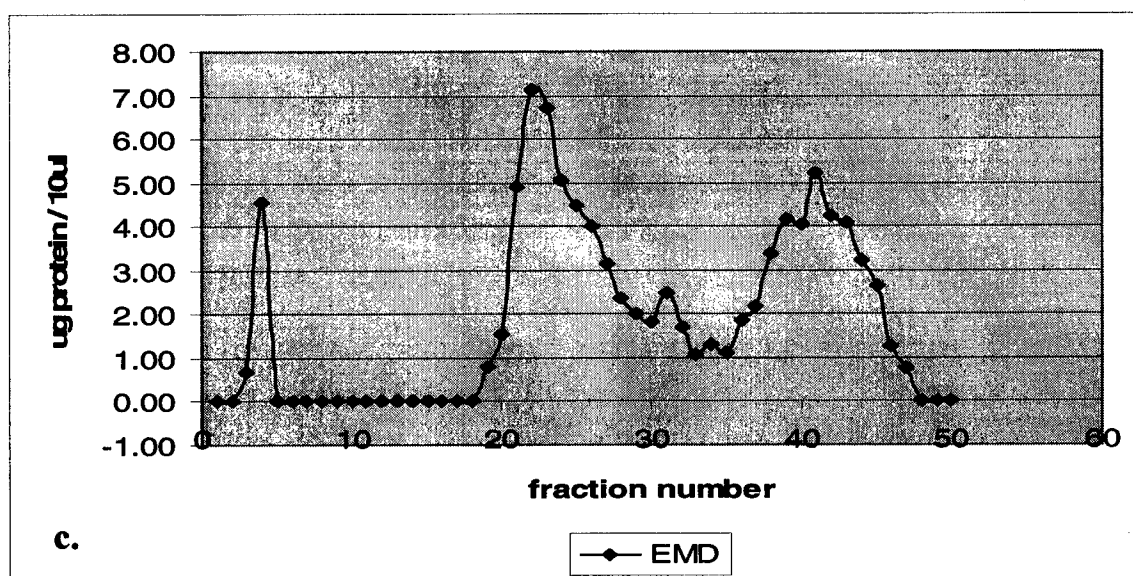
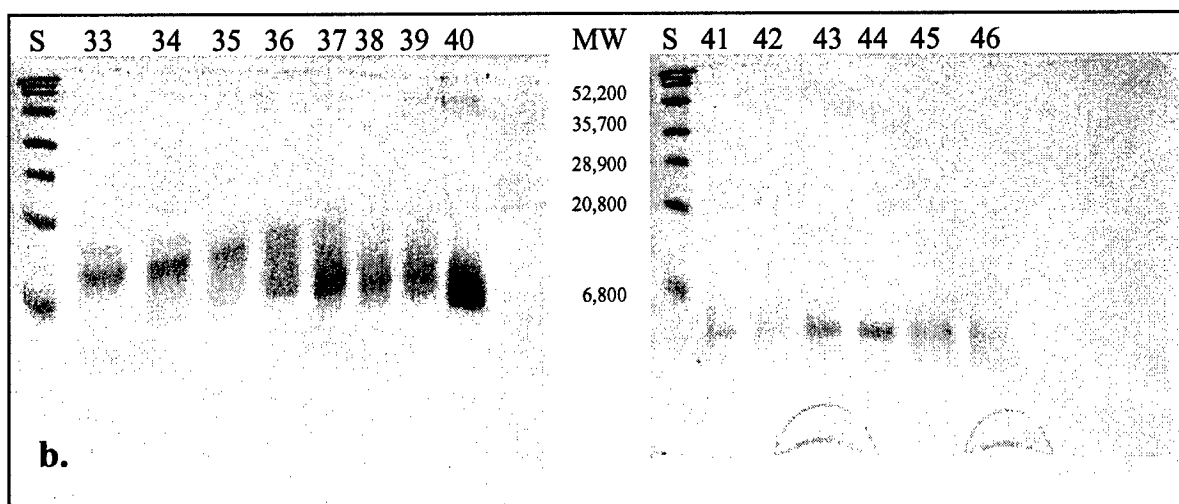
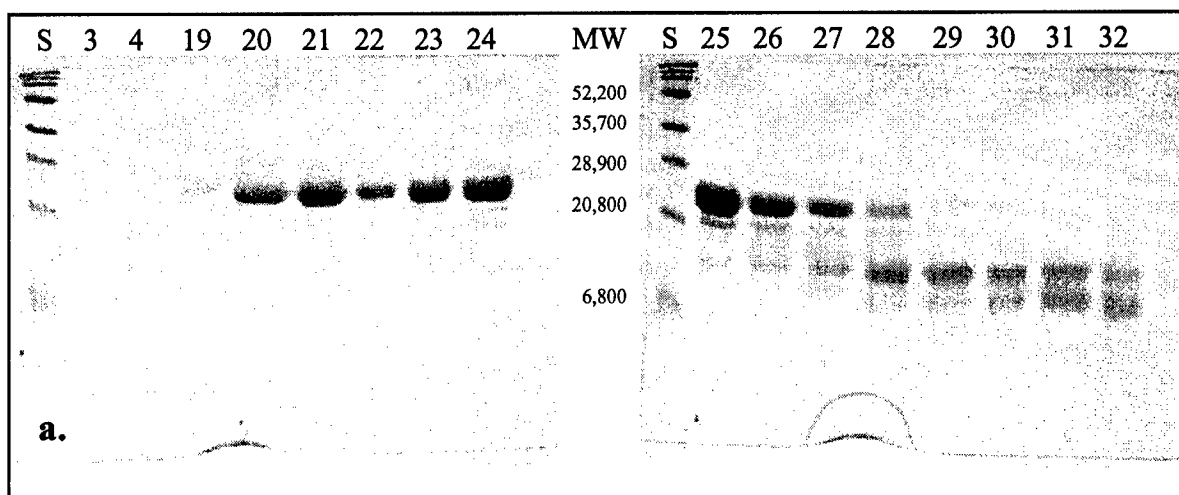


became evident between the 20.8 kDa and 28.9 kDa markers, consistent with where we would expect amelogenin to appear (See Fig. 23a). This band remained prominent through fraction 29 when it began to disappear. In fraction 24 an additional group of light bands began appearing and became more prominent with each following fraction number in the molecular weight range of 6.8-20.8 kDa (See Fig. 23a). The appearance and increase of these protein bands corresponded with the decrease and disappearance of the higher molecular weight band (i.e. amelogenin). From fraction 32-45 there were only 2 light bands evident around the 6.8 kDa marker. These bands appeared to merge into one band in the latter fractions at slightly less than the 6.8 kDa marker (See Fig. 23b). The protein pattern evident in the fractions on the SDS page gel corresponds extremely well to the fractions from the column separation in which the second and third protein peaks appear (See Fig. 23c). It is interesting to note that whereas prefractionated EMD (See Fig. 10) contained a higher molecular weight band, the fractionated EMD contained no such band (See Fig. 23a and 23b).

One potential reason for this to occur is that no protein was detected from the BCA assay on fractions 5-18 in the column used to collect fractions for analysis on the SDS page gels (See Fig. 23c). Therefore, samples from these same fractions (5-18) were not included on the SDS gels (See Fig. 23a). It is possible however, that proteins in this molecular weight region are present in small amounts, but only detectable with a more sensitive assay. The proteins in this molecular weight region would be greater than a molecular weight of 52,200 (See Fig. 10), which would place these proteins between the first and 2nd major protein peaks on the EMD protein separation profile (See Fig. 15), and in the region of alkaline

phosphatase activity. Interestingly enough, this would also be the molecular weight region where BMP's would be expected to fall.

Figure 23. SDS-PAGE Gels of EMD Column Fractions with Molecular Weight Markers. a. No protein bands were evident in any of the fractions from 0-18. A very strong band became evident in fraction 19 between the 20.8 kDa and 28.9 kDa markers, consistent with where amelogenin would appear. In fraction 24 additional bands appeared and became more prominent in later fractions in the molecular weight range of 6.8-20.8 kDa. b. In fractions 32-45 two light bands were evident around the 6.8 kDa marker which merges into one band in latter fractions. c. EMD protein separation profile corresponding to the fractions on the SDS page gel.



V. DISCUSSION AND SUMMARY

With the approval of EMD for clinical use in 1997, a new era began in periodontics, one in which the goal of achieving more predictable and complete regeneration has come one step closer to being realized. The rationale for using enamel matrix proteins is that regeneration could be accomplished by mimicking what occurs during normal tooth development. Unfortunately, the mechanism by which this occurs is still unknown, thus this is quest of much of today's research. Since amelogenin is the main protein in EMD, making up 90% of the composition, it is widely believed that the effects of EMD must be due to amelogenin, but this has not been proven. The goal of this study, to fractionate enamel matrix derivative and determine the extent to which the known activities of proliferation and differentiation, are the result of a single protein component in the mixture (i.e. amelogenin), has been accomplished. In the process, this research contributes two new, important, and exciting pieces of information to the literature. First, the study shows that EMD stimulates angiogenesis in microvascular endothelial cells, and second, that EMD also contains proteolytic activity, information that, to our knowledge, has not been previously reported.

In this study, 30mg of commercially available EMD (Emdogain[®]) was fractionated using a Sephadex G-100 column, into 45 seven ml fractions containing protein that eluted in three distinct peaks. This is in agreement with Iwata *et al.* (2002), who fractionated their own preparation of enamel matrix proteins obtained from the tooth germs of 6 month old

pigs and by using the same method for fractionation also found three distinct protein peaks. The protein separation profile was very similar to that found in the study reported here.

With regard to alkaline phosphatase activity, a marker for cellular differentiation by osteoblasts and osteoprogenitor cells, this study found that when applying both EMD and the column fractions to C2C12 cells that differentiation did occur. This is in agreement with other studies stating that EMD does stimulate cellular differentiation (Schwartz *et al.*, 2000; Ohyama *et al.*, 2002), but opposed to other findings, in which markers for differentiation were not found (Hakki *et al.*, 2001; Chano *et al.*, 2003). In this study, when the alkaline phosphatase activity of the column fractions applied to C2C12 cells was measured and superimposed on the EMD protein separation profile, it was evident that it did not correspond to any of the major protein peaks, but rather, the strongest activity fell between the first and second protein peaks. The same pattern was observed when using ST2 cells as well (data not shown). This partially agrees with the study reported by Iwata *et al.* (2002), who found that the strongest alkaline phosphatase activity of EMD fractions also occurred between the first and second major protein peaks. However, in contrast to our data, they also found alkaline phosphatase activity to be associated with the first protein peak or void volume. The difference could possibly be explained by the difference between the commercial EMD used in our research compared to their enamel matrix protein formulation. It may also be due to the fact that the Iwata study used a mouse bone marrow stromal cell line, ST2 as the test cell. The present study also found that by pre-incubating the column fractions with the decoy receptor noggin, prior to treating C2C12 cells, that alkaline phosphatase activity was inhibited. Again, this is consistent with the Iwata *et al.* (2002) study using ST2 cells. In another recent study by Takayama *et al.* (2005) using C2C12 cells, which normally do not

express core binding factor $\alpha 1$ /Runt-related transcription factor-2 (Cbfa1/Runx2) unless stimulated by BMP-2 (Nishimura *et al.*, 2002), found that EMD stimulated Cbfa1/Runx2 expression in the cells that could also be inhibited by noggin. These studies, together with the results of this study, suggest that the ability of EMD to stimulate cellular differentiation is due to a BMP family member. This is because the activity is both inhibited by noggin (Iwata *et al.*, 2002), and does not correspond to the molecular weight region of the chromatogram associated with amelogenin or any of its fragments. It is interesting to note that one recently hypothesized mechanism of action for EMD is that proposed by Parkar *et al.* (2004), in which gene arrays from PDL cells treated with EMD appeared to up regulate genes coding for growth factors and growth factor receptors while down regulating inflammatory genes. One of the specific genes that is upregulated, is BMP-4, one of three BMP's known to be inhibited by noggin. Because alkaline phosphatase activity stimulated by EMD is inhibited by noggin, this potentially strengthens the argument that the differentiation that occurs is due to a BMP family member, possibly BMP-4. Parkar's *et al.*, (2004) idea of EMD upregulating genes goes a long way towards explaining not only many of the observed effects involving growth factors and/or their receptors (Hoang *et al.*, 2002 ; Lyngstadaas *et al.*, 2001), but also the marked improvement in wound healing that has been observed by many when using EMD.

Another of the genes that appears to be upregulated by EMD is vascular endothelial growth factor precursor (Parkar *et al.*, 2004). With vascularity so essential to wound healing, it follows that an upregulation of this growth factor may stimulate the necessary cellular activities leading to improved wound healing. The results of the present study appear to confirm this finding because when HMVEC cells were stimulated with EMD, an ensuing

increase in proliferation was observed, consistent with similar results reported by Yuan *et al.* (2003) both *in vitro* and in a murine model. A similar increase in proliferative activity was also found to occur in the individual column fractions that corresponded to the major protein peaks, suggesting that the proliferative activity of EMD may be due to amelogenin. This pattern of proliferation was not unique to HMVEC cells. It also occurred in the C2C12 cell line.

Another important event in wound healing besides that of endothelial cell proliferation is angiogenesis. To our knowledge, the angiogenic activity of EMD has not been reported previously in the literature, and like proliferation, corresponded to the major protein peaks, suggestive, once again, of being associated with amelogenin or one of its fragments. Yuan *et al.* (2003), although stating that they looked at angiogenesis, really only reported data on endothelial cell proliferation and chemotaxis, and not angiogenesis. One of the important factors in the formation of new vessels and granulation tissue is vascular endothelial growth factor (VEGF) (Howdieshell *et al.*, 2001). In a study by Mirastschijski *et al.* (2004), they found that VEGF production by fibroblasts was increased five fold and 5.5-fold as compared to control-treated fibroblasts at 3 and 6 days. This appeared to not only increase the formation of granulation tissue, but also made it more resistant to trauma. Several other significant findings related to healing in this same paper, were that wounds appeared to be smaller, epithelialization greater, and healing times decreased when EMD was used compared to controls. If EMD is upregulating precursors to vascular endothelial growth factors as suggested by Parkar *et al.* (2004), it would help explain not only the healing results by Mirastschijski *et al.* (2004), but it also helps explain the link between the proliferative and

angiogenic activities of the HMVEC cells observed in this study, both of which were found to correspond to the major EMD protein peaks.

Another important part of both wound healing and regeneration is the breakdown of type I collagen. In this study proteolytic enzyme activity, or more specifically collagenolytic activity, was found to occur in two regions, in fractions 3-8 and 17-21. The first region was associated with a high molecular weight region in the void volume of the column, possibly consistent with the presence of MMP-2, a matrix metalloproteinase falling into the broad category of gelatinase A, and known to digest amelogenin (Caron *et al.*, 2001; Cotrim *et al.*, 2002). A wound-healing study by Mirastschjski *et al.* (2004) gives further credence to the findings of this study that the collagenolytic activity observed, may be due to MMP-2. They found increased levels and activation of MMP-2 by both endothelial and fibroblast cells when treated with EMD. Although endothelial cells are known to express MMP-2 during wound healing (Mirastschjski *et al.*, 2002), the amount was significantly higher when treated with EMD, suggesting that either MMP-2 is either present in EMD or up-regulated by it. The collagenolytic activity in this higher molecular weight region could also be masking potential alkaline phosphatase or proliferative activities that may be associated with this protein peak by degrading necessary substrates before being activated. To evaluate the possibility of this occurring one would need to rerun the alkaline phosphatase and proliferation assays utilizing an enzyme inhibitor that would allow the activity to be manifest if present.

The second region of collagenolytic activity was associated with a lower molecular weight region consistent with the presence of MMP-20, or enamelysin, another matrix

metalloproteinase known to digest amelogenin (Wang and Moradian-Oldak, 2002). Further research is necessary to specifically characterize the newly discovered metalloproteinase activities and is beyond the scope of this research.

Although individual activities have been separated out and discussed in conjunction with the protein(s) or peaks potentially responsible for the activity, it is important to realize that individual activities do not occur in a vacuum exclusive to one another. Rather, the activities more likely act in concert with each other, and the various cell types and/or receptors in a dynamic environment to stimulate and/or accelerate the regenerative process. Further research to identify the specific BMP's, proteins, and/or amelogenin fragments which are responsible for these activities is necessary before one can hope to understand the interactions that occur which lead to predictable regeneration.

In conclusion, the results of this research have shown that EMD has the ability to stimulate multiple activities in more than one cell type, which is extremely important in the multicellular process of regeneration. The differing and specific biological effects of EMD were also clearly associated with proteins of differing molecular weights, and, therefore, unlikely to be associated with a single protein species (Amelogenin \cong 23 kDa). The proliferative and angiogenic activities may be associated with amelogenin and/or its fragments, while differentiation of osteoprogenitor cells was most likely the result of BMP(s). Finally, we found that EMD contains proteolytic activity, an activity that has not been previously reported in the literature and which merits further investigation.

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VITA

Dwight L. Johnson was born ~~XXXXXXXXXX~~ in Colorado Springs, Colorado to ~~XXXXXXXXXX~~. He graduated as Salutatorian from Clearfield High School in 1986 and attended Brigham Young University in Provo, Utah where he graduated with a Bachelor of Science degree in Chemical Engineering, a Bachelor of Arts degree in Portuguese, and a minor in Chemistry in 1994. Dr. Johnson attended Southern Illinois University School of Dental Medicine where he earned his Doctor of Dental Medicine Degree with honors on June 5, 1999. During his second year of dental school Dr. Johnson received a Health Professions Scholarship from the Air Force, and following graduation, entered active duty military service as a Captain in the United States Air Force (USAF). He completed a 1 year Advanced Education in General Dentistry (AEGD) program at Wright-Patterson Air Force Base in Dayton, Ohio in July of 2000 and then served as a general dentistry officer in the Air Force for two years while stationed at Lajes Field, Azores, Portugal. In June 2002, Dr. Johnson entered the three-year post-doctoral Periodontics program at Wilford Hall Medical Center and the University of Texas Health Science Center at San Antonio, Texas. Upon graduation, he will be assigned as a staff Periodontist and advanced education in general dentistry (AEGD) instructor in Periodontics at Langley AFB in Virginia.

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